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VOLUME 26 NUMBER 2

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

OFFICIAL ORGAN OF BIOLOGICAL STAIN COMMISSION

APRIL 1951

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Contains final 20 pages of 5-year index

Published by
BIOTECH PUBLICATIONS, Geneva, N. Y.

Entered as second class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

ISSUED QUARTERLY

SUBSCRIPTION PRICE $4.50 PER YEAR (U. S. CURRENCY)

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Articles dealing with microscopic technic, or with the nature or uses of biological stains are suitable for publication in STAIN TECHNOLOGY. It is understood that articles submitted have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which must be complete in itself and contain all the important facts brought out in the paper. When possible the abstract should summarize the new methods in such form that a reader can follow the directions without consulting the body of the paper.

All staining formulae must be definite and accurate. The batch or certification number of the stains actually used by the author in such formulae should be included for reference purposes. In case of uncertified stains, not designated by any batch number, one should give as much information as available, i. e. name of manufacturer, approximate date of purchase, etc.

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STAIN TECHNOLOGY

VOLUME 26 APRIL, 1951 NUMBER 2

A UREA SILVER NITRATE METHOD FOR NERVE FIBERS AND NERVE ENDINGS

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Received for publication July 14, 1950

ABSTRACT.—**A silver nitrate stain for nerve fibers and endings applicable to paraffin sections on the slide utilizes the properties of urea to accelerate the procedure and improve the specificity of the stain. After removal of the paraffin the sections are run through absolute, 95% and 80% alcohol and placed for 60–90 minutes at 50–60°C. in: 1% aqueous silver nitrate, 100 ml.; urea, 20–30 g.; 1 g. mercuric cyanide and 1 g. picric acid in 100 ml. of distilled water, 1–3 drops. After the silver bath they are rinsed quickly in 2 changes of distilled water and reduced for 3–5 minutes at 25–30°C. in: water, 100 ml.; sodium sulfite, anhydrous, 10 g.; hydroquinone, 1–2 g.; urea, 20–30 g. They are then washed thoroughly in 4–5 changes of distilled water, passed through graded alcohols into 80% alcohol and examined under the microscope. If nerve fibers are not distinct, the sections are returned to the same urea-silver-nitrate bath for 10–15 minutes, rinsed, reduced, washed and dehydrated as before. This process may be repeated until staining is adequate; then they are dehydrated, cleared, and mounted.**

Nerve fibers show a color range from brown to black; nerve cells from yellow to brown; and the background, depending on the type of tissue and its fixation, from yellow to light brown.

Urea is one of the simplest non-electrolytes known to increase the solubility of coagulated proteins (Laporta[1]); it also increases the diffusion rates of both electrolytes and non-electrolytes and facilitates their penetration into proteins, as shown by Bechhold and Ziegler[2] for gelatin and other proteins (Fischer and Sykes[3]).

---

[1]LAPORTA, M. 1932. Beobachtungen über die Renatusierungen der Proteine. Kolloidzschr., **61**, 376.

[2]BECHHOLD, H., and ZIEGLER, J. 1906. Die Beeinflussbarkeit der Diffusion in Gallerten. Zts. Phys. Chem., **56**, 105.

[3]FISCHER, M. H., and SYKES, A. 1915. Über die nicht-saure und nicht-alkalische Hydration des Proteins. Kolloidz., **16**, 129.

This is a report of attempts to take advantage of these properties of urea to modify the physical state of proteins in tissue sections in order to achieve selective staining of certain tissue elements on reduction of silver nitrate. The method resulting from these empirical trials preferentially stains nerve fibers and nerve endings of the central and peripheral system. This technic, applied to paraffin sections on the slide, gives constant and rapid results and eliminates the need of gold-toning which is essential to most of the shorter silver methods.

Fixation

Selective staining of nervous structures depends to a large extent upon the initial fixation of the tissue. Fixatives which have given good results with this method are:

(1) 25 g. chloral hydrate dissolved in 100 ml. of 50% alcohol;
(2) 20 ml. formaldehyde mixed with 80 ml. of 95% alcohol;
(3) 2 g. ammonium bromide dissolved in 15% aqueous formalin;
(4) 95% or 100% alcohol;
(5) Bouin's fluid.

The first four mixtures have mainly been applied to central nervous tissue and nerve trunks; (2) and (5) to gland and smooth muscle tissue; (1) to striated muscle tissue; and (5) to embryos. Tissues fixed in 10% aqueous formalin have often shown good results, but an excessive staining of connective tissue also has been observed. Solutions containing chromate, chromic and osmic acid, or mercuric bichloride have been found not to be suitable as fixatives.

Method

1. Remove paraffin with xylene and run sections through absolute and 95% alcohol to 80% alcohol.
2. Immerse slides directly from the 80% alcohol for 60–90 minutes at 50–60°C. (place in paraffin oven) in the following urea-silver-nitrate solution:

1% aqueous silver nitrate	100 ml.
Urea	20–30 g.
1 g. mercuric cyanide and 1 g. picric acid in 100 ml. of distilled water	1–3 drops.

3. Rinse quickly in 2 changes of distilled water.
4. Reduce for 3–5 minutes at 25–30°C. (room temperature) in a solution prepared by adding the following reagents in the order indicated:

Distilled water	100 ml.
Sodium sulfite anhydrous	10 g.
Hydroquinone	1–2 g.
Urea	20–30 g.

Agitate the slides gently for about the first 2 minutes.

5. Wash thoroughly in 4 or 5 changes of distilled water.

6. Run sections through graded alcohols to 80% alcohol and examine under the microscope. If the staining is not completed, repeat steps 2, 3, 4, 5, and 6 as often as necessary. The original solutions may be used in this restaining but the time the sections are kept in the urea-silver-nitrate bath (step 2) should be reduced to 10–15 minutes.
7. When staining has been completed, dehydrate through 95% and absolute alcohols, clear in xylene and mount.

Results. Nerve fibers appear in the color range from brown to black, nerve endings usually black, and nerve cells from yellow to brown. The background is usually yellow but its appearance depends not only on the kind of tissue but also on the fixation used.

Comment

For the initial silver nitrate bath slides are placed into the cold urea-silver-nitrate solution which should be made up just before use. The 1% silver nitrate and the mixed solution, containing 1% mercuric cyanide and 1% picric acid may be kept in stock. The reducer can be made up in larger quantity without the urea, which should be added just before use. Since urea chills a solution as it dissolves, the reducer should be warmed up to 30°C. before use; otherwise its action may be insufficient.

The length of time the sections are kept in the initial urea-silver-nitrate bath seems to determine more or less the outcome of the procedure. Therefore, it is advisable to make some trial slides to determine the optimum time.

A concentration of 20% urea in both the silver bath and the reducer was generally employed, but sometimes better staining was obtained by adding 25 or 30 g. per 100 ml. Urea, USP, from several chemical houses has been consistently satisfactory.

The addition of mercuric cyanide and picric acid was found to be essential by empirical trials and seems to improve discreteness of fine nerve fibers and nerve endings.

In the process of silver salt impregnation with subsequent reduction, the urea-silver-nitrate stain is probably very much like other empirical silver technics that have been discussed in recent years by Zon, Davenport, Silver and others.[4]

The role of urea in this staining procedure seems to be complex; it changes the physical state of the tissue and also could possibly have protective action in suppressing the ionization of silver nitrate.

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[4] Zon, L. 1936. The physical chemistry of silver staining. Stain Techn., **11**, 53–67.

Silver, M. L. 1942. Colloidal factors controlling silver staining. Anat. Rec., **82**, 507–29.

Davenport, H. A., and Kline, C. L. 1938. Staining paraffin sections with protargol. Stain Techn., **13**, 147–60.

The chemical and physical factors which account for the affinity of nerve fibers for silver are poorly understood, but they are modified by urea to exaggerate the differential staining. There is the additional benefit that the addition of urea to both the silver nitrate solution and to the reducer by retarding the formation of larger aggregates of silver, results in an extremely fine grain.

Summary

The specificity of silver nitrate staining of nerve tissue is increased by making the reagents up in 20–30% urea solution. The technic, given in detail, is dependable and consistent when applied to paraffin sections.

STAINING APPARENT NUCLEAR MATERIAL IN BACILLUS CEREUS, NEISSERIA CATARRHALIS AND OTHER COCCI

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Received for publication August 8, 1950

ABSTRACT.—**The method of staining involves the principle of reduction and oxidation of some cell component. The reduction is a function of the mordant and the oxidation that of the stain. Three areas of the cells may be differentiated depending upon the condition of the mordant. The wall may remain unstained, while that of the cytoplasm stains a light red and structures within this a deep red. The latter structures only may be stained by proper adjustment of the mordant.**

The proposed technic for staining bacterial cells involves the principle of reduction and oxidation of some cell component. The reducing agent, or mordant, is prepared in large test tubes made of soft glass. New tubes are preferred, and should be washed with soap and rinsed first in water and then in alcohol. The mordant consists of the reaction product of finely powdered iron by alcohol, hematein (technical), and water. The iron and hematein are used in the ratio of approximately one mole of iron to two moles of hematein, though the ratio of the reaction of one to the other is not known; in practice, 0.25 grams of iron, 2.57 grams of hematein and 60–65 ml. distilled water have given good results. The material is placed, in the order named, in a dry test tube, which is stoppered with a cork and placed on a slide warmer having a temperature of 55°–60°C., as measured by a thermometer in water in a beaker resting on the heater. In a few minutes bubbles of hydrogen start rising from the iron-hematein mixture. The tubes are kept on the warm plate until the bubbles cease to appear. The time required may be 48 hours or longer. If the iron cakes in the bottom of the tube during the reaction, it should be broken up with a clean iron wire. When no more bubbles rise from the mixture, the tubes are removed from the slide warmer and set aside while the solution undergoes further changes. The time required for these changes varies from a few days to 2 or 3 months.

The solution is dark blue in color even before it is removed from the heater, and may already be active for *Micrococcus pyogenes* var. *aureus* and other cocci; but usually some time must pass before it will reduce *Bacillus cereus* which is used as a standard for testing the activity of the mordant. When found ready for use it is centrifuged

and the supernatant poured into a clean container and kept tightly stoppered. While the mordant gains activity for *B. cereus* it loses activity for the cocci but may be reactivated for the latter organisms by adding pyrogallol. The amount to be added will depend on the state of the mordant and also on the particular organism to be stained. Some mordants and organisms may not require more than 4 mg. per ml. whereas others may require 20 mg. per ml.

Some difficulty has been had in obtaining the product known technically as *iron by alcohol* in a very finely powdered form. *Iron by hydrogen* may be used provided it is refluxed with acetaldehyde and thoroughly dried. The process may be carried out in a test tube to which the iron and acetaldehyde has been added. The tube is corked tightly and placed in a cup on the slide warmer. When the acetaldehyde boils the vapors condense near the top of the tube and return to the boiling liquid.

The hematein (technical) was obtained from the Eastman Kodak Co., Rochester, N. Y.

The stain is prepared by adding 50 ml. of absolute alcohol, free of traces of aldehyde, to 1.5 g. of rosanaline hydrochloride in a large pyrex test tube. Basic fuchsin which contains a high percentage of rosanaline hydrochloride may be used but in practice the pure chemical has given more satisfactory results. Oxygen is bubbled through the solution for 30 hours or until saturated. This constitutes a stock solution, and is made up for use as follows:

Chloroform	15 ml.
Solution of phenol in chloroform (8 g. in 100 ml.)	3 ml.
Stock solution of dye	3 ml.

A colloid is formed upon the addition of the dye but settles after several hours, leaving a clear red solution. It should be centrifuged before use. Either the stock solution or the made-up dye keeps indefinitely.

For best results, the bacteria to be stained should be grown on glucose agar. A firm agar a few days old is apparently the best medium in terms of the staining process.

The staining procedure is as follows: Smear the organism in a drop of the mordant, which has been placed on a slide. Place the slide immediately on the slide warmer (55°–60°C.), and allow to dry thoroughly. Stain while on the warm plate, either by adding a drop of dye for one or two seconds and blotting off, or by making a very thin film of stain over the smear with a capillary pipette held in a horizontal position. In either case it is not necessary to wash the slides. The results with *B. cereus* may show either the cytoplasm stained a light red and the apparent nuclear material[1] much more deeply

[1]The author's reasons for regarding this central, red-stained material as nuclear in nature are being written up for publication elsewhere.

stained or only the latter stained. In the case of the cocci central bodies only are stained. If *B. cereus* is smeared into the mordant used for the cocci then the walls remain unstained while the cytoplasmic region is a uniform red color. Although no complete survey of bacteria stained by this method has been made, the following organisms have been stained: *Bacillus cereus, Micrococcus pyogenes* vars. *aureus* and *albus, M. perflavis, Neisseria catarrhalis, Streptococcus pyogenes,* and *Strep. lactis.* It is hoped that other organisms will be subjected to this technic in the near future.

DETERIORATION OF NITROCELLULOSE SOLUTIONS CAUSED BY LIGHT

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Received for publication August 10, 1950

ABSTRACT.—**Solutions of *Parlodion* stored in clear glass bottles, and subjected to the usual lighting of laboratory work tables, deteriorate progressively. Blocks of nervous tissue stained by Cajal's formula No. 6 underwent marked fading when embedded in old, light-affected Parlodion solutions. Storage of nitrocellulose, either as a solid or in solution, should be in darkness, in a dark colored bottle, for example.**

During an anatomical study of brains stained with silver nitrate (Cajal's formula No. 6), fading of the stain during nitrocellulose embedding was a frequent occurrence. Fading was sometimes sufficient to be ruinous, and the diffusion of the stain into the surrounding embedding mass was evidenced by a cloud of finely granular precipitate. The stained zone of the embedding mass often attained a thickness of a centimeter or more.

A somewhat similar observation of fading by Golgi silver-dichromate stains was mentioned by Porter and Davenport (1949). The fading occurred in some blocks of stained tissues but not in others and no explanation of the cause of the phenomenon could be given.

After a rather extended search for the cause of apparently unpredictable fading, we concluded that it might be a result of the action of light on exposed nitrocellulose solutions stored in clear glass bottles. Consequently, experiments were designed to test this assumption.

EXPERIMENTAL PROCEDURE

Brains of rabbit and albino rat (100 specimens altogether) were stained according to Cajal's formula No. 6 as follows:

The pieces are fixed for 24 hours in 10% chloral hydrate in distilled water, rapidly washed (1 min.) in distilled water, and transferred to 95% alcohol with 10 drops of strong ammonia water in each 100 ml. for another 24 hours. They are then blotted with filter paper and transferred for 5–6 days to 1½% silver nitrate solution kept in the oven at 35°C. They are washed in distilled water (1 min.)

[1]Fellow of the Rockefeller Foundation on leave of absence from the Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay.

[2]Publication No. 553, Department of Anatomy, Northwestern University Medical School, Chicago, Illinois.

and then reduced 15–20 min. in a 0.5% solution of pyrogallic acid in 7.5% formalin to which 5% by volume of acetone has been added, and reduction completed for 24 hours in a 1% solution of pyrogallic acid in 15% formalin. Dehydration and embedding follows.

The 6% solution of nitrocellulose *(Parlodion,* Mallinckrodt) was prepared in equal parts of absolute alcohol and anhydrous ether (Baker). From this concentrated solution the medium (3%) and the weak (1½%) solutions were prepared just before use. In order to obtain comparable results, the embedding process *per se* was always done in the same manner. The tissues were kept 8 hours in 70% alcohol, 18 hours in 95% alcohol with several changes, 4 hours in absolute alcohol with several changes, and in alcohol-ether mixture for another 4 hours. Twenty-four hours were allowed for each one of the three embedding solutions: weak, medium and thick. Thickening of the final (6%) solution was allowed to take place rather slowly in a loosely-covered Stender dish, with about 15 days as the time for solidification. The solid mass was transferred to 70% alcohol for hardening. Sections were cut at 20μ.

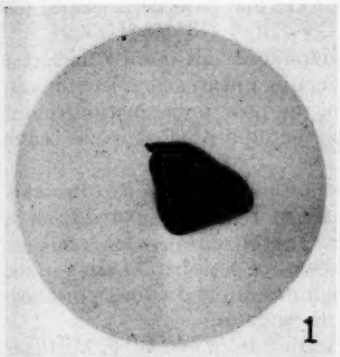
Results

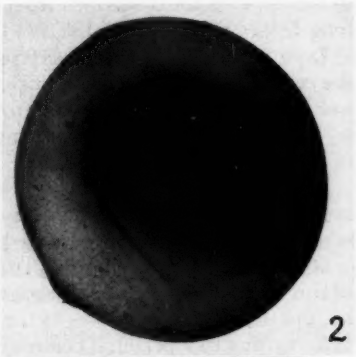
When the embedding was done with a *Parlodion* solution stored in a dark bottle (the same bottle in which the dry product is sold) little or no destaining of the blocks was observed even though the solution was used several months after its preparation. At most, an insignificant ring of silver appeared in the embedding mass around the tissue but no recognizable loss of staining was observed on microscopic examination of the sections.

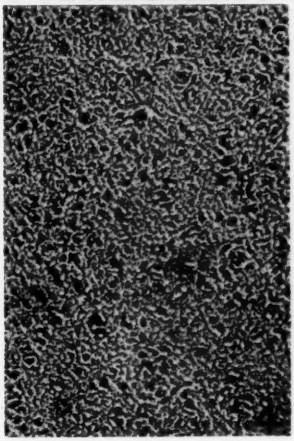
On the other hand, when the embedding was done with solutions stored in clear bottles exposed on the work table to daylight (but not direct sunlight), it was possible to see the beginning of the deleterious action of light on the impregnation after a few weeks of exposure. Even before cutting, this was evidenced by the presence of a heavy ring of silver in the embedding mass around the tissue. In the more extreme examples of fading of the stain, the entire mass became cloudy and darkened until it was impossible to locate visually the position of the tissue.

By doing successive embeddings with the same *Parlodion* solution every 15 days, it was easy to recognize, even macroscopically, the progressively increasing removal of stain by the embedding mass. When this process of diffusion was very marked, the silver even passed from the block into the alcohol used for hardening and was deposited on the sides of the container.

With microscopic examination, the early fading appears in the section as a peripheral ring where the nerve fibers are no longer impregnated or are very pale. This is in contrast with the deeper layers where the fiber impregnation is good, and is somewhat similar







FIGS. 1 and 2 (at natural size) show two pieces of nervous tissue stained with Cajal's silver nitrate formula No. 6 and embedded in nitrocellulose. The embedding solutions in both cases were prepared 6 months before use, but that used for specimen 1 was stored in a dark bottle while that for specimen 2 was exposed to light. In specimen 1, the embedding mass is clear and the darkly-stained tissue is sharply seen. However, in specimen 2 there is a heavy diffusion of the silver from the tissue into the nitrocellulose. This forms dark rings and black spots in the embedding mass. The outline of the tissue is barely discernable.

FIGS. 3 and 4 (270×) depict the same regions (the external Stria of Baillarger in area 2 of cerebral cortex of albino rat) in sections which were impregnated and embedded in the same way. In the first case, the nitrocellulose used was freshly prepared, while in the second, a nitrocellulose solution deteriorated by several months exposure to light was employed. Observe the heavy fiber density in Fig. 3, in contrast to the complete absence of stained fibers in Fig. 4. In the latter, the large neurons still retain some silver. This destaining of the fibers is associated with the gross aspect seen in Fig. 2.

to the appearance of sections from blocks that have been washed too long before being reduced.

Even when the fading is very pronounced, one usually finds that the central part of the tissue block retains a nearly complete impregnation. This is quite different from the poor impregnation caused by incomplete penetration, in which the stain is palest in the center of the block.

Figures 1–4 show different degrees of fading caused by nitrocellulose solutions which had been exposed to light as compared to those which were either freshly prepared or kept in dark bottles.

It is obvious that even a small amount of peripheral fading in the brains used for some special studies, for example cortico-architectonics, can defeat the objectives of the investigator.

According to a personal communication from Mr. W. F. Michener of the Mallinckrodt Chemical Works, dry *Parlodion* is packaged in black bottles, since it is light sensitive and breaks down unless protected in that manner. He has pointed out that it is also light sensitive after it has been dissolved in an alcohol-ether mixture. Our results indicate clearly that nitrocellulose solutions acted upon by light cause a destaining or fading of silver-stained nervous tissue, and that this does not occur when they are stored in darkness.

It is suggested that this deterioration of nitrocellulose with exposure to light may effect the quality of the embedding mass itself by making it more friable after gelation. Casual observations on old solutions used to embed unstained tissues indicate that blocks of poor cutting quality resulted when such light-deteriorated solutions were used.

The authors wish to thank Dr. H. A. Davenport for his advice on this problem.

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Ramón y Cajal, S., and de Castro, F. 1933. Técnica micrográfica del sistema nervioso. Tipografía, artística, Madrid. *See paragraph 117.*

A COMBINED CONNECTIVE TISSUE STAIN FOR ELASTIN, RETICULIN AND COLLAGEN

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Received for publication August 10, 1950

ABSTRACT.—**A staining technique for demonstrating reticulin, elastin and collagen in the same tissue sections is based upon the use of a silver stain for reticulin, orcein for elastin and picro-anilin blue or fast green for collagen and other tissue structures.**

Many connective tissue stains have been devised. Some are utilized to distinguish connective tissue structures from muscle or epithelium, while others are specifically designed to demonstrate one type of connective tissue fiber such as collagen, elastin or reticulin. In order to demonstrate all of these different connective tissue fibers in the same microsection we have combined a familiar stain for elastin, reticulin and collagen. Orcein[1] reveals the elastic fibers, Foot's silver stain[2] impregnates the reticulin and picro-anilin blue[3] or fast green and picric acid differentiates the collagen. This combined stain is performed without undue difficulty and while it presents no dramatic color contrasts under low-power magnification it beautifully distinguishes the connective tissue fibers under moderate to high magnification. This stain has been of great value in studying the morphology of tuberculous and other inflammatory reactions, diseases of the cardiovascular system, collagen and reticulin formation in vitamin C deficiency, the development of connective tissue fibrils in the embryo and the invasion of carcinomas into preexisting structures.

PREPARATION OF STAINING SOLUTIONS

Solution 1. Foot's modification of Hortega's silver carbonate method. To 10 ml. of a 10% aqueous solution of silver nitrate add 10 ml. of a saturated aqueous solution of lithium carbonate. Wash the precipitate 3 times with distilled water, allowing the precipitate to settle each time and pouring off the supernatant fluid. Add 25 ml. of distilled water. Almost dissolve the precipitate with 28% ammonia water added drop by drop while shaking. Make up to 100 ml. with 95% ethyl alcohol. Filter and warm to 50°C. for 15 minutes before using.

[1]See Lillie (1948) pp. 198–200; Mallory (1938) pp. 171–2; Conn and Darrow (1949) p. IB_5–16.

[2]See Mallory (1938) pp. 165–7; Conn and Darrow (1949) p. IB_5–20.

[3]See Lillie (1948) p. 191.

Solution 2. Orcein (C.I. No. 1242),[4] 1 g., dissolved in 100 ml. 70% ethyl alcohol. Add 1 ml. concentrated hydrochloric acid.

Solution 3. Dissolve 0.1 g. anilin blue (C.I. No. 707) in 100 ml. saturated aqueous picric acid.

Solution 4. Dissolve 0.2 g. fast green FCF in 100 ml. saturated aqueous picric acid.

All solutions with the exception of the silver carbonate may be kept for a period of several weeks, with a slight increase in the staining time of the picric acid solution, and with a slight decrease in the staining time of the orcein solution. When aging makes the staining of the orcein solution too intense, the original staining qualities may be regained by adding 1% hydrochloric-acid-alcohol in the ratio of 1 vol. acid to 20 vol. staining solution.

Method

Formalin fixation, paraffin embedding, sections cut at 4 or 5 μ.

1.	Xylene	5 minutes
2.	Alcohol, 100% ethyl	2 minutes
3.	0.5% collodion (ether-alcohol)	5 minutes

Drain one minute. This step helps to prevent sections from becoming detached from the slide during the silver impregnation. The collodion is removed by the absolute alcohol in the final differentiation and clearing steps.

4.	Alcohol, 80%	5 minutes
5.	Rinse in tap water	
6.	0.25% potassium permanganate, aqueous	5 minutes
7.	Rinse in tap water	
8.	5% Oxalic acid	10 minutes
9.	Rinse in tap water, followed by distilled water	
10.	Stain in silver ammonium carbonate (Solution 1) in incubator (50°C.)	10–15 minutes
11.	Rinse in distilled water	
12.	20% neutral aqueous formalin	5 minutes
13.	Rinse in tap water	
14.	Tone in 1:500 aqueous gold chloride	5 minutes
15.	Rinse in tap water	
16.	Sodium thiosulfate, 5%	2 minutes
17.	Rinse in tap water	
18.	Stain in orcein (Solution 2) in incubator (37°C.) for 10–15 minutes or one hour or more at room temperature	
19.	Rinse in 70% alcohol, followed by tap water	

[4] An uncertified sample from Hartman-Leddon was used primarily in this work; but a certified lot, FOr.–2, from the National Aniline, seemed to give similar results.

20. Picro-anilin blue (Solution 3)	20–40	seconds
Or: Picro-fast green FCF (Solution 4)	10–20	seconds
21. Alcohol, 95%	6–8	seconds
22. Alcohol, absolute		briefly
23. Alcohol-xylene		until clear
24 & 25. Xylene		until clear

Results

Picro-Anilin Blue:

Elastic fibers	Red-tan to red-brown
Collagen	Blue
Reticulum	Black
Muscle	Blue-green
Nuclei	Tan to brown
Erythrocytes	Yellowish-tan
Cytoplasm	Pale blue

Picro-Fast Green:

Elastic fibers	Orange-red to red-brown
Collagen	Blue-green
Reticulum	Black
Muscle	Green
Nuclei	Tan to brown
Erythrocytes	Yellow to orange yellow
Cytoplasm	Light green

If a more intense nuclear staining is desired, an iron hematoxylin stain may be inserted following Step 19. Weigert's iron hematoxylin is satisfactory. A stain for acid-fast bacilli may be incorporated by staining with Ziehl-Neelsen's carbol-fuchsin for 10–15 minutes on a steaming rack following Step 17. It is not necessary to decolorize following this stain, as the hydrochloric acid in the orcein stain decolorizes sufficiently. The picric acid stains have shorter staining times following the acid-fast stain. Opaque and deeply stained microsections are nearly always due either to thick sections or excessive staining with picro-anilin blue or picro-fast green.

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THE USE OF ACETONE-BENZENE INFILTRATION IN HISTOLOGICAL TECHNIC

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Received for publication September 8, 1950

In a study of several amphibian blastulae and gastrulae *(Rana pipiens)* it was noticed that with the employment of ordinary infiltration and embedding technics, if dehydration and infiltration did not take place slowly, the blastocoel roof would almost always collapse. The amount of time during which embryos of this type must be processed in these technics in order to maintain the blastocoel roof in its normal morphological position, is rather lengthy; and the technics, themselves, are tedious and drawn out.[2] In addition, material treated thus, often sections poorly, the yolky, vegetal hemisphere being somewhat brittle and dry because of poor paraffin infiltration.

A method which will dehydrate and infiltrate rapidly, and which will, in addition, preserve the normal blastular and gastrular features of the embryo is ideal because of the time saved, and also, because of the quality of the results obtained. Such a method involves the use of acetone, acetone-benzene mixtures, and benzene as infiltrating agents. The method consists of the following:

1. Fix embryos in Smith's fluid for 12 to 24 hours.
2. Wash in running tap water overnight to remove the formalin.
3. Remove the jelly membranes by rolling the *fixed* embryos over filter paper with a #5 watchmaker's forceps or an ordinary dissecting needle.
4. Place the denuded embryos in 70% alcohol for a few minutes. Embryos may be stored at this point.
5. Remove to 95% alcohol for one-half hour.
6. Immerse in acetone for an hour.
7. Place in a 1:1 mixture of acetone-benzene for 15 minutes. *The benzene should be thiophene-free.*
8. Clear in benzene for 15 to 30 minutes. The yellow vegetal hemisphere usually darkens when the embryo is sufficiently cleared.
9. Infiltrate with paraffin for one-half to three quarters of an hour. Paraffin with a melting point of 56°–58°C. has been shown to be

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[2]KING, R. L., and SLIFER, E. H. 1933. Grasshopper eggs and the paraffin method. Science, **78**, 366.

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excellent for this purpose, although lower melting point paraffin can be used.

Embryos infiltrated by this method show little or no distortion due to shrinkage. Furthermore, the blastocoel roof remains unwrinkled and does not collapse during the dehydration and infiltration process. Such embryos have been shown to section well at 10μ, and if necessary, even at 7μ for nuclear studies. The yolk in the vegetal hemisphere, and the dorsal lip region of the embryo remain intact.

This quick method of acetone-benzene infiltration has been employed successfully in conjunction with the methyl-green-pyronin staining of the nucleus and cytoplasm in amphibian blastulae and gastrulae,[3] and also, with the Feulgen technic for the demonstration of desoxyribonucleic acid in the nucleus.[4]

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[3]Rosa, C. G. 1950. Triethyl phosphate in the methyl-green-pyronin staining technic. Stain Techn., **25**, 165.

[4]Rosa, C. G. 1950. The cytological distribution of desoxyribose nucleic acid in O_2-pressure blocked embryos of *Rana pipiens*. Unpublished data.

HISTOCHEMICAL TESTS FOR POLYPHENOLS IN PLANT TISSUES

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Received for publication September 19, 1950

Abstract.—**A satisfactory histochemical test for polyphenols in fresh plant tissues is described. The test is based upon a colorimetric method for phenolics using a nitrous acid reaction. Certain catechol derivatives are characterized by the formation of an intense cherry-red in fresh sections of plant tissues with the reagents used. The red color is sufficiently intense to be readily observed microscopically at 100×. Results obtained with other tests also are described.**

Polyphenols in many plant tissues become oxidized to polymeric, dark red or brown compounds (phlobaphenes), which sometimes are microscopically visible in the cell contents of fresh sections. Histochemical detection of these naturally occurring polyphenols is difficult because few reagents that react with them to form characteristic color compounds are adaptable to histological methods. Also, the natural enzymatic browning may not be sufficiently intense for ready detection microscopically. Lison's (1931) methods for specific reactions of ortho- and para-polyphenols in animal tissues are not applicable to fresh sections of plant tissues; they are tedious and dependent upon exacting technics (see also Cowdry, 1943, for references on azo- and indo-reactions by Lison).

Common, unspecific tests for tannins consist of treatment of sections with ferric chloride solutions followed with a few drops of dilute sodium carbonate or other base (Johansen, 1940; Rawlins, 1933). Usually a blue-green color or a precipitate is formed but not all phenolics give such a reaction and the test may be influenced by other materials present. In addition, excess ferric chloride must be thoroughly washed out of the section; otherwise, muddy brown precipitates of iron hydroxide result when the base is added. Zirkle (1932) has used various precipitants in killing agents for fixing tannins *in situ*. This technic is useful for the preparation of permanent sections, and Johansen (1940) prescribes 2% ferrous sulfate in 10% formalin for the fixing reagent. Dark precipitates are formed in the vacuoles containing tannins. In general, however, precipitating reagents such as iron salts for phenolics do not provide as sensitive tests as are chemically possible with qualitative color reactions which are more specific for differentiation of phenolics.

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Pectic materials also form dark precipitates with iron salts in plant tissues, possibly because the iron-tannin complex is adsorbed in pectinaceous areas. Ferric chloride used with tannic acid is an excellent stain for the middle lamellae (Foster, 1934) and also stains pectic materials in cellulose walls. Johansen's method with ferrous sulfate also stains middle lamellae and cell walls, as found in using this fixing solution on fresh sections of apple tissues. When ferric chloride instead of ferrous sulfate was used with the formalin it was found that the vacuoles of many cells retained the natural color of ferric chloride indefinitely and even after mounting in balsam, while the cell walls and middle lamellae were stained from black to gray.

Histochemical localization of chlorogenic acid and other phenolics by treatment of plant tissues with either ammonia or dilute ammonium hydroxide and exposure to air has been reported by Politis (1947, 1948). As stated by Politis, chlorogenic acid in alkaline solution turns from yellow to green in the presence of oxygen, and the green changes to red-violet in acid solution. As observed here, the green color formed upon sufficient oxidation in ammonium hydroxide turns pink to lavender in acid solution and the color change is reversed on addition of alkali. Some flavones and other catechol tannins gave yellow to red-brown colors with ammonium hydroxide but generally faded or did not change to a red-lavender when acid was added.

In use of this ammonia test on fresh fruit sections it was found that the yellow and brown colors faded with the addition of acid. The green color also was obtained in some tissue regions and appeared to be due to chlorogenic acid. In not all instances, however, could a definite change of the green to red-lavender be accomplished, but in these cases the green was not persistent.

In sections of apple tissue treated with ammonium hyroxide the green appeared in the cells of the skin region and in the phloem areas with sufficient intensity to be microscopically visible. Yellow and red-brown colors also developed in or near the same areas and there was evidence of interference with the green reaction by these other colors. Fruit tissues exposed to ammonia vapor for about an hour developed such dark brown coloration that the yellow and green reactions could not be detected.

A new and satisfactory histochemical test for phenolics in fresh sections of plant tissues was developed at this laboratory by a simple adaptation of a nitrous acid reaction for phenolics. In this reaction a nitroso derivative is formed; upon the addition of a base, as in the test described here, a colored compound, the salt of the derivative, results. This test was originally applied by Hoepfner (1932) for the detection of chlorogenic acid in coffee bean extracts. Gonzales Gomez and San Martin Casamada (1942) pointed out that the reagents used by Hoepfner also gave color reactions with other phenolics. Vorsatz

(1942) modified Hoepfner's test and found it useful for differentiating a number of phenolics in solution. This modification is as follows:

To 50 ml. of unknown phenol solution are added (1) 10 ml. 10% sodium nitrite, (2) 10 ml. 20% urea (as a stabilizer), (3) 10 ml. 10% acetic acid, and (4) after 3 minutes, 20 ml. 2*N* sodium hydroxide. Urea was not used in the original test.

The Hoepfner-Vorsatz and the ammonium hydroxide tests were used on several phenolics and other materials in aqueous solutions of about 0.01% for visual comparisons with the following results:

Substance	Test	
	Hoepfner-Vorsatz	*Politis*
Catechol	Dark cherry-red persisting 1+ hours.	Amber changing to red-brown in several hours.
Chlorogenic acid	As with catechol.	Greenish yellow changing to green; red-lavender in acid solution (reversible).
Hydroquinone	Red-brown, lighter brown on standing several minutes.	Iodine-red to brown.
Orcinol	Very dark red-brown.	Orange brown becoming red-brown on standing.
Para-phenylene diamine	Red-orange, becoming turbid red-brown.	Colorless.
Phenol	Light yellow.	Bluish on standing.
Phloroglucinol	Yellow, changing to light brown.	Light rose-lavender, changing to red brown in several hours.
Pyrogallic acid	Brick-red, changing to dark brown.	Red-brown.
Quercetin	Amber to brownish.	Yellow changing to light red-brown in several hours.
Quercitrin	Reddish brown.	Yellow changing to orange in several hours.
Resorcinol	Orange changing to brown.	Green-yellow changing to brown in several hours.
Rutin	Reddish brown.	Yellow changing to light brown in several hours.
Tannic acid	Light yellow to brown.	Iodine-red to brown.
Vanillin	Light yellow.	Colorless.

It was found that catechol and chlorogenic acid dilutions of 1 part in 250,000 gave readily visible color formation with the Hoepfner-Vorsatz test.

Since catechol derivatives undoubtedly occur as mixtures in plant tissues, various combinations of the pure compounds in solution were tested with the Hoepfner-Vorsatz reagents. Mixtures of both catechol and chlorogenic acid with less than equal amounts of quercetin, quercitrin, and rutin gave a cherry-red which showed little visible change toward a red-brown. When larger amounts of each flavone were used in mixtures with either catechol or chlorogenic acid, and initial cherry-red color changed to a clear red-brown or dark orange which persisted an hour or more.

Adaptation of the Hoepfner-Vorsatz test to a histological method consisted simply of adding drops of the reagents to fresh sections in proportion to the amounts previously described and in sufficient quantity to cover the section. In apple tissue, the skin area, phloem and associated parenchyma, and many of the storage parenchyma cells developed a dark cherry-red which slowly changed to a dark brick-red upon standing about an hour. There was no pronounced diffusion of color from intact cells and no other colors were observed.

Similar color reactions were obtained in sections of fresh peach fruit. In these the color formation was more generally distributed throughout all parenchyma tissue than with apples where the most intense red developed in the cells associated with phloem and skin. In pear sections the contents of the sclerids (stone cells), core parenchyma, and the outermost region of the fleshy tissue showed an intense cherry-red with this test; in the intervening area of fleshy tissues pronounced color formation occurred only in scattered parenchyma cells and in the sclerids. Color formation in sections of full-grown, green fruits generally were more intense than those obtained with sections of ripe fruit, but the same zonal patterns prevailed.

The regions in which the cherry-red color formed corresponded in general to areas in which enzymatic browning was obtained when fresh sections were exposed to air several hours. These same areas also provided a positive reaction for polyphenolase when dilute catechol was added to fresh sections. They likewise coincided with the regions in which iron-tannin precipitates could be detected in the cell contents when sections were treated with ferric chloride. However, the nitrous acid reaction obviously was a more sensitive test, since intense cherry-red developed in areas in which the iron-tannin precipitates were faint or doubtful and which showed at least faint enzymatic browning.

Sections of juniper and pine leaves, among other Gymnospermous tissues in which phlobaphenes were readily observed, also were tested. In all cases the red color of the Hoepfner-Vorsatz reaction was observed in areas where iron-tannin complexes were obtained with ferric chloride. Phlobaphenes developed an intense cherry-red, and colloidal or granular materials in the vacuoles of some cells likewise were intense cherry-red with the nitrous acid reaction.

A number of chemical color tests for phenols have been reported in the literature and several of these were tried as histochemical tests. Among these, 2–6 dichloroquinonechloroimide (Porteous and Williams, 1949); dimethyl–p–phenylenediamine (Houghton and Pelly, 1937), phosphomolybdic acid (Brauer, 1926), and ammonium molybdate (Quastel, 1931) were the principal reagents used. In most instances the tests were not satisfactory for histological method; they either lacked selectivity under histological conditions or the color

formations were not sufficiently intense to be readily observed microscopically.

Vinson (1910) and others have used ethyl nitrite in a nitrous acid reaction for studying tannin changes in ripening fruits. The relationship of the nitrous acid reaction, and of other reactions of phenolics to dye chemistry is discussed in most modern organic chemistry texts, such as that by Karrer (1938).

The use of iron salts, potassium bichromate, osmium tetroxide with potassium nitrate, and other metallic salts as precipitants for tannins dates back many years. Nierenstein (1935) has reviewed the history of these tests and their introduction into botanical literature by DeVries, Sanio, and others. The botanical advantages of precipitating tests of this sort lie in the fact that they permit an exact intracellular location and have thus been found excellent for certain cytological purposes. Unfortunately, "tannin" is so loosely used that it is applied to nearly all naturally occurring soluble and colloidal phenolics, phlobaphenes, and (on the basis of non-specific tests) to various other materials in plant cells with which metallic salts form precipitates.

Phenolics are not necessarily associated with vacuoles, but frequently are found in them. Phenolics appear to be among the materials involved in the formation of vacuoles in meristematic tissues in many plants. Some of the relationships of vacuoles to other cell parts and tannins in plant meristems are well described by Zirkle (1932).

Although no exhaustive investigation has been made, the results described here for the Hoepfner-Vorsatz test indicate that it may be used for histochemical localization of certain phenolics in fresh plant tissues. The test described by Politis also may be useful for differentiating chlorogenic acid from flavones and other phenolics when the results are carefully interpreted. The usefulness of these tests on meristems has not been investigated, but it would appear that they might have some utility in augmenting other technics. Choice of any histochemical test naturally should be governed by the particular objective in view.

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ON THE COMPOSITION OF "VISCOL" AND THE EMPLOYMENT OF GUM ARABIC FOR MOUNTING MEDIA

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Received for publication October 9, 1950

ABSTRACT.—**"Viscol", a water soluble permanent mounting medium hardening through evaporation of water under the cover glass, has been analyzed. It proves to consist of a mixture of gum arabic, glycerol, phenol and water and is especially suitable for simple botanical preparations. The use of gum arabic for hardening permanent mounting media is reviewed. Instead of a glycerol-phenol-water mixture a lactophenol, potassium acetate or zinc chloride solution mixed with gum arabic may be used for a permanent mounting medium. However, gum arabic contains calcium, magnesium and potassium ions giving crystals with the solutions mentioned. In the case of lactophenol and potassium acetate, the calcium and magnesium ions must be removed beforehand, which is done by precipitation with sodium or potassium carbonate. In the case of zinc chloride the potassium ions must be removed, which is done by dialysis with zinc chloride. It is pointed out that the same principles may be used for a great variety of different mounting media.**

In botanical investigations preliminary inspections of simple preparations (a leaf of a moss, a hand section through the stem or leaf of a vascular plant, a spore preparation of a fungus, a piece of the thallus of an alga etc.) are often carried out, and it may be found desirable to preserve the preparation. A transfer to the usual mounting media (Canada balsam, euparal, glycerol jelly etc.) usually takes much time and labor and may destroy the preparations.

In 1935 and 1936 Dr. J. Amann announced a new mounting medium called "Viscol". He found that old preparations usually had been destroyed or damaged for some reason or other; only preparations kept in lactophenol or in zinc chloride (with or without iodide) had persisted. But lactophenol and zinc chloride solutions are fluid; thus the preparations must be kept in a horizontal position, and cleaning the cover glasses before a renewed inspection is dangerous.

Amann analyzed the requirements of an ideal mounting medium for the use of botanists for simple preparations. It should be soluble in water, it should harden in time under the cover glass, the refractive index of the medium should be quite different from the tissue to be investigated, it should cause no distortion of the cells, the important structures like chloroplasts and oil bodies should be

preserved and the preparations should not be destroyed or damaged over a period of many years. These are difficult requirements, not all of which can easily be fulfilled by one mounting medium.

The "Viscol" invented by Amann and sold through the Drougeries Reunies in Lausanne fulfills many of the above requirements. It forms a liquid soluble in water which after a few days hardens under the cover glass. The preparations are, according to Dr. Ammann, well preserved for many years, an observation which is confirmed by the present author.

In 1939 Dr. Amann died and left no information as to the manufacture of "Viscol". Through the courtesy of the Drougeries Reunies I received 20 tubes for analysis.

"Viscol" is a limpid fluid, somewhat more viscous than glycerol and smells strongly of phenol. In acid solution "Viscol" is oxidized rapidly by permanganate, and if absolute alcohol is added a white fluffy precipitate is obtained. According to Dr. Amann (1936, p. 23) "Viscol" is a phenyl derivative of viscose in a solution of glycerol. Thus "Viscol" probably is a solution of an unknown substance in glycerol, water and phenol. "Viscol" hardens through the loss of water and may be softened again by water. The unknown substance may be purified through repeated precipitations with alcohol.

Viscose preparations are obtained by treating cellulose with caustic soda and afterwards with CS_2. Viscose gives characteristic reactions (black precipitate with lead salts), but no such reactions could be found with "Viscol".

The term viscose has sometimes been used also for other cellulose derivatives. There is the possibility that the unknown substance is a phenyl derivative of cellulose formed e.g. by etherization of the OH-groups through phenol. Similar cellulose ethers like the methyl, ethyl, benzyl and oxyacetic acid ethers are soluble in water. However, even large reference books say nothing about a phenyl ether of cellulose.

The well known cellulose ethers mentioned above may have some value as mounting media. They, however, give gelatinous solutions at a concentration of 2–5% and the concentration must be lower than 1% for making fluids. Thus considerable contraction by evaporation of water is necessary to make the mixture hard, and this makes the cellulose ethers less suited for mounting media. Better results may be obtained through partial hydrolysis of the chains of the cellulose ethers, but the process was found to be difficult to control and no good results were obtained. The contraction of "Viscol" on evaporation of water under the cover glass is comparatively small.

A chemical analysis of the unknown substance of "Viscol" was kindly carried out by mag. Maalfrid Halvorsen. It proved to contain 2.3% ash on ignition (as carbonates). It also contained C, O and

H in ratios very close to those of a polysaccharide. It contained no ethyl or methyl groups. The analysis shows that the substance is not a phenyl derivative of cellulose since the introduction of sufficient phenyl-groups in the cellulose chains to make the phenyl ether water soluble, certainly would appreciably have affected the ratio of C and H to O.

The data at hand makes it most probable that the unknown substance is a salt of a polyuronic acid. Gum arabic is known to be a salt of a polyuronic acid with potassium, magnesium and calcium. These elements were all found in the ash from "Viscol" and the percentage of ash also corresponds well with gum arabic. A solution of gum arabic in water and the unknown substance of "Viscol" in water behave in the same manner in all respects tested by me. There is no doubt that the unknown substance of Viscol is gum arabic.

A suitable medium for mounting is made in the following proportions:

80 g. gum arabic
40 g. glycerol
90 g. water
20 g. phenol

The gum arabic is dissolved in the glycerol-water mixture and phenol added afterwards. I have kept preparations in this mixture for a year and they behave in the same way as those mounted in "Viscol". Preparations ready to use may be obtained in tin tubes through the Drougeries Reunies, Lausanne, Switzerland.

If instead of the glycerol-phenol-water mixture, Amann's lactophenol is employed, crystals are formed after some time. This is probably due to the calcium and magnesium ions giving crystals of calcium or magnesium lactate when the water is evaporated. Thus these ions should be removed before mixing the gum arabic with lactophenol. This may be done through precipitation of the magnesium and calcium ions by sodium carbonate. The following procedure is recommended.

Dissolve 100 g. gum arabic in 150 ml. water and heat to 100°C.
Dissolve 3 g. sodium carbonate in 10 ml. water and mix the solutions.
A white precipitate is formed in one or two days. The solution is freed from the precipitate through decantation.
To 100 g. gum arabic thus prepared 50 g. lactophenol is added.

According to my experience no crystals are formed by this medium. For preparations of fungi and lichens it may be mixed with cotton blue for staining.

Gum arabic mixtures have for a long time been used for mounting media; e.g. the Berlese mixture (15 g. chloral hydrate, 15 g. gum arabic, 10 g., i.e. 7 ml., syrup of liquid glucose, 5 g. acetic acid, and 20 g. water); Farrants's medium (4 g. gum arabic, 2 g. glycerol, and

4 g. water); as well as some other mixtures (see The Microtomists Vade-mecum 1946 paragraphs 458–63). Lately Highman (1946) has introduced two new gum arabic syrups for demonstrating amyloids in sections and at last a diethylene glycol glycerol syrup gum has been introduced by Lillie and Laskey (see Greco 1950 p. 12) but the composition has not yet been published.

The lactophenol gum arabic is very useful especially for preparations of vascular plants and may replace the usual glycerol gelatin for most purposes. However, preparations from mosses and especially fungi become too transparent in the course of some months. This is probably due to the glycerol penetrating the walls of the hyphae and giving them a refractive index very close to the medium. The same is the case with other glycerol mixtures. Thus a new medium with a different refractive index and without glycerol is required.

Preparations of fungi are often kept in a solution of potassium acetate. A possible permanent mounting medium might be made by a suitable mixture of potassium acetate and gum arabic in water. However, such a mixture will form crystals after some time, due to the calcium and magnesium ions forming calcium or magnesium acetate which crystallizes. Thus the gum arabic must be freed from calcium and magnesium ions beforehand. This may easily be done by precipitation of the said ions through potassium carbonate. The following procedure for making a suitable mounting medium for preparations of fungi and lichens and mosses is recommended:

Dissolve 100 g. gum arabic in 150 ml. water and heat to 100°C.

Add 2 g. potassium carbonate in 10 ml. water, stir the mixture and permit the precipitate to settle overnight. The solution is freed from the precipitate by decantation; and the following added:

4 g. glacial acetic acid
45 g. potassium acetate
A small amount of cupric acetate.

Preparations of lichens have been kept for a year in this mixture without formation of any crystals, the spores (except for some *Caloplaca* species) and hyphae are distinct and the green color of the gonidia is preserved. The mixture causes some plasmolysis of the gonidia. The plasmolysis may be reduced through fixation beforehand and by dilution of the medium permitting the concentration gradually to increase through evaporation of water.

A corresponding mixture with zinc chloride instead of potassium acetate has also been made. If gum arabic and zinc chloride are mixed and permitted to harden by evaporation of water, crystals are formed which indubitably consist of potassium chloride. Thus the potassium ions and preferably also the magnesium and calcium ions should be removed and replaced by zinc ions. This may be done by dialysis. The following procedure is recommended: Dissolve 100 g.

gum arabic in 200 ml. water and add 10 g. zinc chloride. The solution is permitted to dialyse overnight through approx. 100 sq. cm. of parchment paper. Another 10 g. of zinc chloride is added, and the process repeated. The water is evaporated in a large porcelain dish by normal temperature. To 100 g. gum arabic thus prepared, is added: 150 ml. water; 65 g. zinc chloride; a little cupric chloride; as much hydrochloric acid as is necessary to make the solution clear. The mixture is also useful for preparations of lichens.

In principle it is thus possible to make a permanent solid mounting medium from any salt solution which will not crystallize under normal conditions provided it contains no ions precipitating or otherwise reacting with gum arabic. Thus there is a way open for a series of different mounting media meeting many special requirements. The greatest danger is the contraction of the medium if much water is permitted to evaporate, thus the ratio between gum arabic, salt and water should be such that at normal conditions the medium on evaporation of water has the character of a jelly, sufficiently weak to avoid distortion of the cells.

I have tested the preparations against freezing by putting them into a refrigerator, but no harm could be observed.

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HANDLING SMALL NUMBERS OF MARINE EGGS THROUGH DEHYDRATION AND EMBEDDING

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Received for publication October 19, 1950

ABSTRACT.—**A method is described whereby small numbers of eggs can be embedded compactly. Handling through reagents is achieved in 6 mm. glass tubing having a linen mesh affixed to one end, up to and including paraffin. The egg mass is then obtained and reembedded in a pre-cut paraffin block in which a well has been drilled. Perfect seal of the two masses is achieved with a hot needle. Some advantages of the method are described.**

Difficulty has always attended the problem of obtaining serial sections of marine eggs starting with relatively small amounts of material. In our own studies, the eggs were destined for subsequent examination with ultraviolet, so that pre-staining them in any way in order to render them more visible was not possible. By the method which is described below, the main difficulties of handling the eggs and embedding them in a suitable manner (compact distribution over a small area) were circumvented.

Three-inch lengths of standard 6 mm. pyrex glass tubing are cut, and both ends are fire polished lightly in such a way as to avoid narrowing the bore. Over one end, a small square piece of fine linen or bolting cloth is stretched and held in place by two turns of fine copper wire. The eggs, together with the fixing fluid in which they are suspended, are introduced through the top by means of a drawn out medicine dropper, and a narrow piece of paper on which identification numbers are written is wedged at the top. About 15 such tubes can be conveniently held together by rubber bands and handled together thereafter. While tubes are being filled and labelled, those already prepared are kept wet at all times. This is achieved by padding the bottom of a glass with a thin layer of absorbent cotton and then adding a suitable reagent until the level reaches about 1½ inches. The tubes are then immersed in the liquid open end up. This glass is then used for all the subsequent procedures, but a number of such glasses can be prepared if preferred, each containing a different solution. While carrying the eggs through a given reagent, the tubes should be lifted out of the liquid and the reagent allowed to run out through the mesh at the bottom of the tube. The eggs accumulate against the mesh. The tubes are then lowered rapidly back into the reagent. This pushes the egg mass up and allows very thorough agitation. These operations are repeated a number of times in each solution, the liquid being allowed to run out before transfer to the next reagent. When the eggs are well infiltrated with paraffin (enough changes should be used, and sufficient agitation should be carried out as described above to insure complete

removal of the clearing agent) the tubes are lifted out of the hot paraffin and lowered about ⅔ the height of the paraffin level into ice cold water. When the paraffin has set, cold water can be added until its level in the glass is above the level of paraffin in the tubes. After setting, the tubes are allowed to dry thoroughly at room temperature. Once dry, the copper wire is removed and the linen or bolting cloth is carefully removed. The eggs remain in the glass tube. The tubes are then *gently* heated in the small flame of a microburner, most of the heat being applied away from the egg mass. Every few seconds, one blows gently through the free end of the tube. When the proper amount of heat has been applied, the paraffin inside the tube will melt in contact with the glass and a cylinder of paraffin will be blown out, the front part of which consists of packed eggs. Overheating, of course, must be avoided.

Following this, paraffin of the same kind is poured and cut into small blocks 1″ × ¾″ × ¾″ and affixed to wooden blocks. This is inserted into the microtome and the front surface is shaved until it is flat. The block is then removed from the microtome and, with the help of a small blade scalpel or similar instrument, a small well of about 7 mm. in diameter is drilled in the center of the block to a depth of about 5 or 6 mm. The egg mass is now cut off from the front portion of the paraffin cylinder and placed into the well of the larger paraffin block. Reembedding is now carried out by strongly heating a steel needle and directing it, while still very hot, into the body of the paraffin block near the border of the well. The heat of the needle should be sufficient to melt the paraffin around it and allow it to flow into the well and fuse with the egg mass. This operation is repeated at a few points along the circumference of the well. Near points where the needle has been inserted, the egg plug will be melted, and some of the eggs will be displaced by convection currents. Even if these eggs prove to be damaged by the heat (they rarely are) the star-like distribution pattern that results is of invaluable help in studying serial sections; indeed, since the pattern is asymmetrical, it is very easy to tell the left or right end of a ribbon or even the location of an individual egg. After cooling, the blocks can be trimmed to the desired size and ribbons obtained in the usual manner.

The method described insures a compact distribution of material in a small area. It can be used conceivably for a number of small organisms.

A MODIFIED FREEZING-DRYING APPARATUS FOR TISSUES[1]

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Received for publication September 2, 1950

ABSTRACT.—**The preparation of tissues by the freezing-drying technic is preferred for many histochemical studies because of the rapid fixation, avoidance of deleterious action of chemical fixation and extraction by aqueous and lipid solvents in fixation, dehydration and clearing; to facilitate this procedure a freezing-drying apparatus has been constructed which permits cutting of sections within five hours after the fresh tissue is obtained. Liquid nitrogen provides a most efficient moisture trap and in conjunction with a heating element provides any desired temperature down to −80°C. during tissue drying. Paraffin infiltration is started without disassembling the equipment and completed in a few minutes. Most stains and histochemical reactions for enzyme and other substances can be applied directly to sections of frozen-dried tissue cut from the same blocks.**

Histochemical and cytochemical observations should be made on tissues resembling as nearly as possible their living state. The freezing-drying technic for preparing tissues (Gersh, 1932, 1948; Simpson, 1941) permits the preparation of thin sections of tissues frozen instantaneously and dried in the frozen state without exposure to the usual histologic solvents or protein precipitants. The instrument to be described provides a simple, rapid, reliable and inexpensive method (Stowell) of preparing frozen-dried tissues which may overcome objections which have been made to other equipment (Scott and Williams, 1936; Taylor, 1944; Wang and Grossman, 1949).

This instrument (Fig. 1 and 2) consists of a mechanical forepump and a water-cooled oil diffusion pump which gave an effective vacuum of 10^{-4} mm. of mercury or below. The frozen tissue is supported in a basket (M) with movable partitions to accommodate specimens of different size. The tissue holder (M) is surrounded by a glass tube (P) which is immersed in a Dewar flask (H) containing 4 liters of liquid nitrogen to cool the tissue and provide a most efficient moisture trap (P). Baffle plates (L) prevent water molecules from escaping the trap (P) surrounding the tissue. A heating element situated in the support (O) is adjusted by the rheostat (F) and the temperature is measured by a thermocouple under the same conditions as the tissue (N), by means of the potentiometer indicator (D). The tissue support (O)

[1]Aided by grants from the National Cancer Institute, Atomic Energy Commission and American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

and baffles (L) are supported by a small glass tube (I) with the copper and constantine thermocouple wires (K) traversing the outside and tissue heating element wires (J) inside. This glass tube (I) is suspended by a spring to permit free movement and reduce breakage. Earlier models of this apparatus made largely of glass were frequently broken, and the use of metal parts, except where visibility or low heat conductivity are needed, as presently employed, is recommended. The glass drying tube (P) surrounding the tissue is sealed by the silicon rubber gasket (G) and requires no lubricant. Atmospheric pressure seals the rubber against the surfaces of the glass and metal tubes. The glass tube is easily removed after air at atmospheric pressure is admitted through the metal stopcock (E) in the top of the stainless steel tube which connects the diffusion pump with the drying chamber. If greater capacity were needed, two or more tubes could be attached to the diffusion pump.

Since the mean free path of water molecules would be about 6 cm. at 10^{-3} mm. pressure, 60 cm. at 10^{-4} and 600 cm. at 10^{-5}, there is no advantage in maintaining vacuums much lower than 10^{-3} mm. of mercury. The fact that water molecules from the frozen tissue have only a few centimeters direct path to travel before they are stopped by a most efficient trap, the high pumping speed, low volume to be evacuated, wide diameter and short length of the system and absence of constrictions and many turns in the system account for the more rapid drying time than is achieved with other systems. A moisture trap at the temperature of liquid nitrogen leaves only 10^{-23} mg. of water per liter of gas in contrast to the 10^{-5} mg. left after drying with phosphorus pentoxide (Strong, 1939). Liquid nitrogen is safer to handle than liquid air and may cause less trouble than phosphorus pentoxide.

Thin slices of tissue cut preferably less than 1 mm. thick are straightened on aluminum foil and plunged rapidly into isopentane cooled below −150°C. by liquid nitrogen. The use of aluminum foil does not delay tissue cooling appreciably and keeps the tissue flat so that numerous adjacent sections can be cut from blocks even larger than 1 cm. by 0.5 cm. The frozen tissue is transferred from the isopentane to the tissue holder in a flask containing dry ice where the aluminum foil is removed from the tissue. Critical factors in preparing tissue are thinness of the tissue blocks to facilitate rapid freezing, rapid freezing of tissue to a temperature below −150°C. and avoidance of warming tissue above −30°C. until it is dried. To avoid warming of tissue by air currents or contact with objects, specimens are covered with crushed dry ice after placing them in the tissue holder (M), the tissue support (O) is cooled with dry ice and the glass drying tube (P) is pre-cooled with liquid nitrogen before the tissue basket (M) is transferred to the support (O). The Dewar flask (H) of liquid nitrogen is elevated so that it surrounds the glass drying tube. In 15 to 20 minutes after turning on both pumps a vacuum

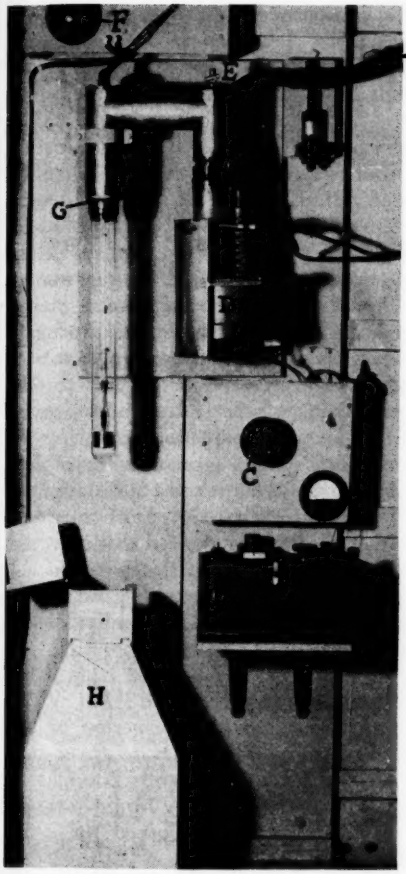

Fig. 1

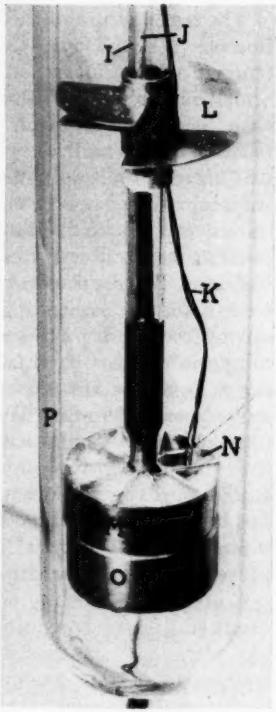

Fig. 2

FIG. 1.—Arrangement of freezing-drying apparatus.
A — Rubber tubing containing vacuum gauge, leading to forepump.
B — VMF 50 diffusion pump.
C — Rheostat and ammeter for diffusion pump heater.
D — Potentiometer temperature indicator.
E — Stopcock.
F — Rheostat for regulating heating element for tissue basket support.
G — Silicone rubber gasket.
H — Dewar flask for liquid nitrogen.

FIG. 2.—Lower end of drying tube.
I — Glass support tube.
J — Wires for heating element.
K — Thermocouple wires.
L — Baffle plates.
M — Tissue basket.
N — Thermocouple basket.
O — Tissue basket support.
P — Tissue drying tube.

below 10^{-3} mm. is reached, as measured by the disappearance of glow from the discharge of an inexpensive Tesla spark coil on the glass drying tube.

The temperature of the tissue is read on the thermocouple potentiometer (D) and adjusted to −30°C. or any other desired colder temperature by means of the rheostat (F). The tissue temperature remains reasonably constant after equilibrium is reached. When the tissue is dry the diffusion pump only is turned off and the rheostat (F) adjusted to melt the paraffin in the bottom of the tissue holder (M) on which the specimens rest. As soon as the paraffin melts, the stopcock (E) is opened, the forepump stopped, the drying tube removed and the partially infiltrated tissue transferred to other melted paraffin preparatory to embedding. A brief paraffin infiltration is sufficient. For routine purposes the total elapsed time from cutting fresh tissues to embedding need not exceed five hours with the use of this freezing-drying equipment and technic. If desired, one group of specimens can be run during the day and another during the night. One group of 20 or more blocks may be frozen and dried with a loss of only 500 ml. of liquid nitrogen. Results are as satisfactory as obtained by drying up to five to ten days with some other eqiupment.

Frozen-dried tissues can be sectioned at any desired thickness and straightened on slides without use of fluids. After removal of embedding media they can be stained with most routine and special stains and histochemical reactions. If carbowax embedding is employed instead of paraffin, fat stains could also be employed.

Costs of constructing such apparatus range upward from a few hundred dollars depending upon parts employed and labor. Working plans and specifications of materials for construction of this equipment may be obtained by sending one dollar to cover costs to Medical Division, University of Kansas Research Foundation, 39th & Rainbow Blvd., Kansas City, Kansas. Where liquid nitrogen is readily available, many specimens can be prepared for a few dollars.

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ZINC CHROMATE MODIFICATION OF THE GOLGI TECHNIC[1]

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Received for publication October 30, 1950

ABSTRACT.—**A modification of the Golgi technic is described in which the reaction takes place in well fixed formalin material. Thin slices (whole sections of adult monkey, cat and rat cerebrum) 2 to 3 mm. thick, from brains fixed 3 to 4 months in 10% formalin, are chromated for two days in 3 g. of zinc chromate dissolved in 98 ml. of distilled water and 2 ml. of formic acid. Slices are then removed, blotted dry and immersed, suspended by a thread, in 0.75% silver nitrate solution for two days. Solution should be changed after the first day. After silvering, the slices are dehydrated rapidly (total time about one hour) in 95% and absolute alcohol, placed in xylene 10 minutes, in low melting point paraffin 10 minutes and embedded in low melting point paraffin. Only surface infiltration is necessary since sections are cut 90 to 100 μ. Sections are collected in 95% alcohol, dehydrated in absolute alcohol, cleared in several changes of xylene and mounted in Fisher's Permount. Results with fetal and new born material were not good.**

Several observers including Durig (1895), Fish (1895), Lachi (1895) and Kopsch (1896) have recommended the use of formalin instead of, or in addition to, the osmic acid in the osmium-bichromate mixture of the rapid Golgi method. Brookover (1910) at times procured good results on adult brains with the rapid process of Golgi's method by a preliminary fixation for 5 to 12 hours in 4% formalin neutralized with lithium carbonate or with ammonia. Hoyer (1894), however, appears to be the first to indicate that the method of Golgi works in material first fixed in formalin, and Bolton (1898) reported good results in brains of cats and half grown kittens placed whole in formalin from five weeks to five months and in human material placed whole in 5% formalin for two months to five months. In the modification of the Golgi method to be outlined here the reaction is obtained in a well fixed formalin material.

PROCEDURE

Brains are perfused and fixed in 10% formalin. The optimum fixation time has not been satisfactorily determined. However, it

[1]This investigation was supported by a research grant from the National Institute of Health, U. S. Public Health Service.

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has been our experience that material fixed for two months or less has given decidedly inferior results; whereas, material fixed for one to two years has given excellent results. In one instance human cerebellar cortex fixed for seven years resulted in rich impregnation. As to the relative merits of fixation by perfusion and fixation by immersion we have no data. All brains of laboratory animals processed by this method have been fixed by perfusion. True, the human cerebellar cortex alluded to was fixed by immersion. Cerebellar cortex, however, is superficial in position and creased by regular furrows between the folia. These factors undoubtedly make it possible for fixing agents to penetrate this structure more rapidly than deeper lying structures. This is especially true in the brains of larger mammals. If a study of the relative effectiveness of these two methods were to be made, we would suggest that for comparison some deeper structure, such as the basal ganglia, be chosen.

In most Golgi methods fixing and chromating are one and the same step, and successful execution of the technic necessitates slicing the fresh brain. Often it is difficult to remove well orientated thin slices in the unfixed brain. An advantage of the present procedure is that large thin slices can be cut prior to chromating since previous formalin fixation imparts the proper consistency to the whole brain. A simple macrotome can be contrived and regular and even slices of the whole hemisphere can be cut 2 to 3 mm. thick. This has further advantages: the orientation of the section is maintained, a complete series can be cut and the wider area being processed increases the possibilities of encountering a region in which the reaction will occur.

The individual slices are chromated for two days in the following mixture: To 3 g. of zinc chromate in 48 ml. of distilled water, add 2 ml. of formic acid and stir until all the chromate goes into solution. Then add 50 ml. of distilled water. In like manner a solution containing 6 g. of zinc chromate can be made, only in this case it is necessary to use 4 ml. of formic acid. We have used both concentrations of the chromate solution and, of late, are tending to use the stronger concentration more frequently.

The chromated slices are removed and blotted dry. A thread is passed through each slice and the slice suspended in a solution of 0.75% silver nitrate. This allows all free surfaces to contact the silvering solution. A large quantity of silver solution should be used. The following day the solution becomes turbid, and the slice should be transferred to a fresh solution. Silvering is completed in about 48 hours. Both before and after the last silvering it is well to cleanse the surface of the slice of excess silver chromate crystals. Distilled water, absorbent paper and a camel hair brush can be used for this purpose. The surfaces are brushed, rather vigorously, and the crystals are wiped onto the absorbent paper. If the crystals are soft and pli-

able, much like gold foil, there is a great possibility that the reaction has been successful. The process of cleansing the surface of the slice can be continued subsequently between and after the changes in alcohol and xylene.

Since a Golgi study requires thick sections in order to follow the processes of nervous elements as far as possible, infiltration and embedding in the ordinary way are not necessary. The slices are rapidly put through two changes of 95% alcohol and two changes of absolute alcohol (the total time required for all these changes is about one hour), then placed in xylene for about ten minutes, low melting point paraffin for about ten minutes, and then quickly embedded. The paraffin thus clings to the surface of the tissue and gives the block requisite rigidity. The tissue itself is of such a consistency that sections 90 to 100μ are cut with ease. As the sections are cut, they are collected in a dish of 95% alcohol and kept in serial order. They should be mounted a short time later. It should be remembered also that once cutting has been commenced it should be continued until the block is finished, since the tissue is not infiltrated with paraffin. If for any reason it is necessary to interrupt the cutting for a short period of time a coating of melted paraffin can be applied to the exposed surface to prevent the block from drying.

The cut sections are passed through absolute alcohol and then several changes of xylene. When the sections are passed through xylene the adherent rim of paraffin is dissolved and crystals of silver chromate separate from the free surfaces and crevices of the sections. It is thus necessary to change and filter the xylene solutions frequently to avoid picking up excessive crystals.

Sections mounted in balsam under a cover slip deteriorate rapidly. This was the experience of the older anatomists and we can verify it from our own experience. On the other hand sections mounted in balsam without a cover slip are irregular and are often difficult to study. We have had some success in mounting them in a synthetic mounting medium (Fisher's Permount). Sections are covered with several coats of Permount and allowed to harden for several days to a week. The hardened surface of Permount is then moistened slightly with toluene and a cover slip is applied. The slide is placed on a warming oven and flat lead weights are allowed to rest on the surface of the cover slip for a few hours to flatten and even the bond formed between the cover slip and the mounting medium.

Some Results

In adult monkeys, cats and rats the modification of the Golgi method outlined above has given some rather excellent preparations, especially in the cerebral and cerebellar cortices, the brainstem reticular formation, the globus pallidus, the putamen, the caudate and amygdaloid nuclei and in a few instances in the thalamus and

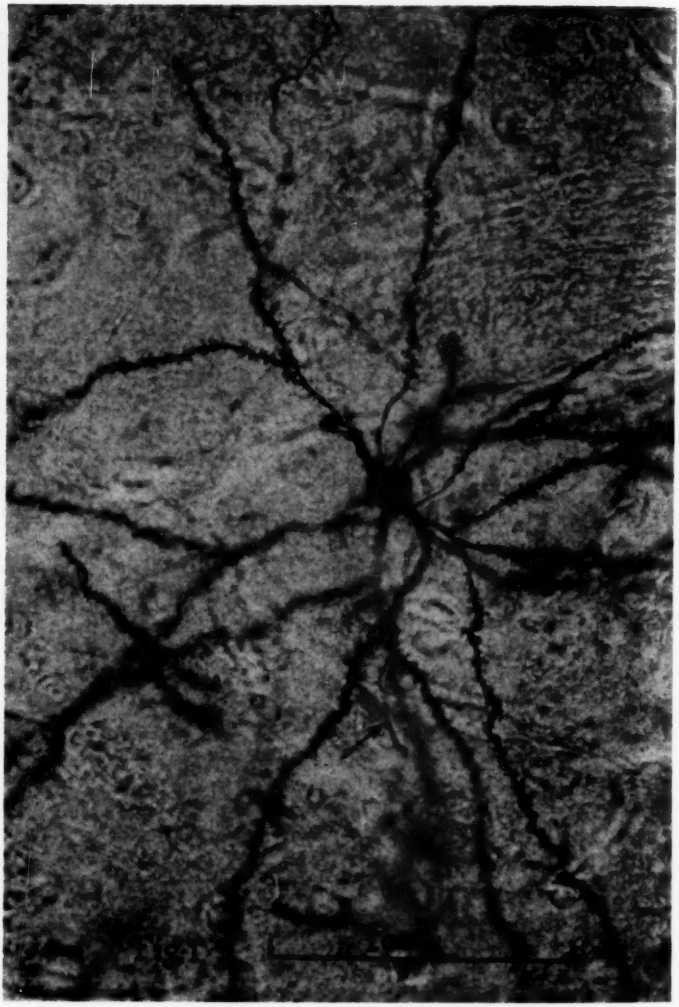

FIG. 1. — From caudate nucleus of adult monkey. Note dendritic spines. Arrow points to afferent fiber which synapses on adjacent dendrite.

hypothalamus. Particularly instructive have been cerebellar sections in which it has been possible to see basket cells, Golgi cells, and granule cells, complete with dendrites and axis cylinders, in addition to the details of mossy fibers, parallel fibers and occasional climbing fibers. The ramifications of Purkinje cell dendrites together with their dendritic spines have been clearly shown and in some preparations it has been possible to follow Purkinje cell oxones and their recurrent collaterals.

Fig. 1 shows a cell in the caudate nucleus of an adult monkey, *Macaca mulatta*. Some of the dendrites are not in sharp focus and show only in shadowy outline. However, the dendritic spines can be clearly seen on the dendrites which are in focus. The arrow points to an afferent fiber which can be followed to its synapse with the adjacent dendrite.

Attempts to impregnate new born and embryonic brains by this method have been most disappointing and we do not recommend it for this purpose. Apparently, formaldehyde methods are unsuitable for embryonic brains (Lee, 1937). Bolton (1898) reported failure in embryonic brains following formalin fixation. Likewise, Porter and Davenport (1949) were unable to obtain good results in fetal material with their method in which material is first placed in a mixture of formalin and silver nitrate.

Discussion

Infiltration and embedding in the ordinary way are not necessary since sections are cut from 90 to 100 μ; this has a time-saving advantage. Ramón y Cajal's material was never infiltrated (Ramón y Cajal, 1895), for it was the opinion of the older authors that prolonged treatment in alcohol had a deleterious effect on the impregnation. Such effects due to treatment in alcohol or xylene could not be found by Porter and Davenport (1949) though they did note a fading of the stain in the periphery of the blocks of specimens embedded in nitrocellulose. However, Hill (1896) prolonged the treatment in alcoholic celloidin up to four days without any perceptible deterioration.

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SOFTENING OF HARD TISSUE FOR SECTIONING

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Received for publication October 9, 1950

Abstract.—**A method for softening refractory paraffin-embedded specimens by trimming the block to just expose the tissue and soaking in solutions of wetting agents (detergents) is recommended. Paraffin blocks are soaked several hours to overnight in 1% aqueous solutions of either new Dreft, sodium lauryl sulfate, Aerosol OT, Glim, or Joy. Excellent sections of rat skin, cartilage, wood and chitin were obtained after soaking. Ribboning and spreading were improved. The method did not improve sectioning of tissue embedded in nitrocellulose when the blocks were stored in 70% alcohol before cutting.**

Tissues are often encountered which are refractory to sectioning after paraffin embedding. The same is true, but to a lesser extent, of specimens embedded in nitrocellulose. The inability to secure sections from skin of albino rats recently led us to investigate the status of softening of embedded tissue.

After trimming the block to expose the specimens, Couch (1930) soaked paraffin-embedded leaves for several days (young leaves) to several weeks (older leaves) in 95% ethyl alcohol containing enough carbol-fuchsin to give it a pink tint, and then in water for 2–24 hours prior to sectioning. Slifer and King (1933) exposed grasshopper eggs to 4% phenol in 80% alcohol for 24 hours before embedding, and then, after making paraffin blocks and exposing the tissue, soaked them in water for 24–48 hours. Cutuly and Cutuly (1934) noticed that rat suprarenals would section equally as well without phenol treatment, when soaked in water alone after embedding. They found that immersion for several days to a week was needed. Johansen (1940), Sass (1940), and Ball (1941) recommended soaking of embedded plant tissue in water to achieve softening. Tahmisian and Slifer (1942) also trimmed the block to expose the tissue, and immersed it in water for at least 24 hours. Baker (1941) used successfully a mixture of 9 parts 60% ethanol and 1 part glycerol for softening paraffin-embedded specimens, but Reeve (1943) found no improvement in the sectioning of nitrocellulose-embedded plant material after soaking in glycerol-alcohol mixtures.

Our attempts to soften paraffin-embedded rat skin by soaking in distilled or tap water produced only superficial softening when the time of immersion was 24 hours, but a mixture of 1 part glycerol and 2 parts distilled water softened almost the entire block in a similar period.

Since the difference in the action of the plain water and the

[1]Publication No. 563 from the Department of Anatomy.

glycerol solution seemed to be the result of a wetting effect which led to the migration of water molecules through the dehydrated protein of the tissue, it was decided to experiment with solutions of various surface-active, or wetting agents (detergents). It was thought that the addition of any one of several detergents to the water would materially decrease the time of softening, and also provide better infiltration of water into the tissue.

Experimental Procedure

In order to compare the effects of various surface-active compounds, 1% aqueous solutions in distilled water of the following detergents were prepared: 1) new Dreft (product of Proctor and Gamble), 2) sodium lauryl sulfate (old Dreft, purified), 3) Glim (product of B. T. Babbitt, Inc.), 4) Joy (product of Proctor and Gamble), 5) Aerosol OT (American Cyanamid Company). Additional solutions were: 6) 0.1% Glim, 7) 0.01% Glim, and 8) 1% dilution of liquid green soap.

Skin of white rat, mammalian cartilage, wood, and a chitinous insect (large beetle) were selected for study, as representative of tissue types which commonly cause difficulty in sectioning. Several fixing fluids were used, and the time of fixation with one (Zenker-acetic) was varied, to determine whether undue hardening might result from prolonged fixation.

Tissue	Fixation
Rat skin, 8×10×3 mm.	Zenker-acetic, 2 hrs., 4 hrs., 6 hrs., 8 hrs., 12 hrs., 24 hrs; and Formalin, 10%
Cartilage, 15×15×3 mm.	Formalin, 10%
Wood	
Maple, green, 8 mm. diam.	Keefe's; and modified Bouin (containing trichloracetic acid, 1/4% by volume, instead of acetic)
Willow, green, 6 mm. diam.	Keefe's; modified Bouin
Birch, seasoned, 8 mm. cube	No fixation
Cucumber, 2 cm. long	Keefe's; modified Bouin
Leaves, from ash tree	Keefe's; modified Bouin
Maize, immature ear, 3 cm. long	Keefe's; modified Bouin
Triangular reed, 1 cm. side	Keefe's; modified Bouin
Beetle, 2.5 cm. long	Gilson's

Prior to fixation, the beetles were transected between the thorax and abdomen, and the two parts were cut midsagittaly. Whenever practicable, adjacent tissues from the same source were compared.

Dehydration and clearing were carried out through graded alcohols to absolute, and thence to xylene. One beetle was cleared with normal butyl alcohol after 95% ethyl, omitting absolute and xylene. Tissue which was to be embedded in low viscosity nitrocellulose was first placed in equal parts of absolute ethyl alcohol and anhydrous ether.

To determine the hardness of the tissue before softening, paraffin sections were attempted at 6 and 10μ. After 2–3 days storage in 70% alcohol, test tissues embedded in nitrocellulose were cut at 15 and

25μ. Any sections or fragments obtained were mounted, and served as untreated controls. Sectioning also exposed the tissue, thereby preparing it for subsequent softening.

After the control sections were cut, each paraffin or nitrocellulose block was placed in one of the several solutions listed, and allowed to remain overnight (16–20 hours). A control block of each tissue was placed in distilled water for an equal time. Sections were again cut the following day.

A. PARAFFIN SECTIONS

Before treatment, sections of rat skin fragmented badly and were unserviceable. After soaking, ribbons of excellent sections were obtained at 6 and 10μ. Tissue soaked in distilled water and a solution of green soap, however, yielded good sections for only about a millimeter into the block, while specimens treated with the detergent solutions were softened throughout their entire depth. When the sections were mounted with albumin adhesive, those obtained after soaking in detergent spread more evenly and quickly than those immersed in water or soap. Subsequent staining revealed well preserved histological detail, with no evidence of distortion or dissociation of tissue elements.

The gratifying success achieved by soaking blocks of rat skin in detergent solutions was duplicated with cartilage, wood, and chitin. Cartilage cut smoothly, without fragmentation or tearing. All specimens of wood were sectioned with equal facility either with the grain or across the grain. Staining was more uniform in the softened material than in the unsoftened. After soaking, sections of the chitinous beetles were greatly superior to those obtained from the untreated blocks. Anatomic relations were preserved, organs were intact, and even egg masses sectioned well. The exoskeleton presented no difficulty, and in no way interfered with cutting at 6μ. After softening, no difference in ease of sectioning could be discerned between the beetle cleared with normal butyl alcohol and those cleared with xylene, although better sections could be cut from the former before soaking.

The five brands of detergents tested were equally efficacious in softening the embedded tissue. The results obtained seem to indicate that the specific type of wetting agent used is not of primary importance. They all afford a relatively rapid method for rehydration of tissues, thereby returning them to a condition which approximates that existing before dehydration. The presence of a firm paraffin matrix throughout the interior of the tissue is essential for satisfactory sectioning, since no amount of softening will enable one to cut good sections on a specimen which is poorly embedded.

B. NITROCELLULOSE SECTIONS

When tissues were embedded in low viscosity nitrocellulose and stored in 70% alcohol for 2–3 days before sectioning, a softening by subsequent soaking in detergent solutions was not noticeable. During the period of storage, the tissue presumably came to an equilibri-

um with the water in the alcohol, and a partial rehydration occurred. Additional hydration did not produce further softening.

Discussion

Our experiment with the time of fixation in Zenker-acetic agreed with the generally acknowledged fact that exposure to acidified dichromate, chromic acid, or mercuric chloride should not be unnecessarily long. It was found that when the time of fixation was limited to no more than 6 hours, other factors being constant, objectionable hardening of rat skin was somewhat less than it was after fixation for longer periods.

Alcoholic dehydration always causes some hardening of tissues. Removal of unbound water from a specimen frequently makes it brittle, but this is less after the use of normal butyl alcohol, which indicates that most of the hardening may be due to the absolute alcohol itself. Clearing with xylene is known to render specimens refractory to sectioning, but it was used in our experiments because it is a popular reagent in many laboratories.

Still another potential source of hardening is prolonged heating in the paraffin oven, although this source has probably been overrated. Proper infiltration should not be sacrificed for the sake of the chance of minimizing hardening. In the course of our experiments, some tissues were left overnight in the oven to insure adequate infiltration. When later sectioned, after softening, there was no evidence of any deleterious effect of the long embedding time.

Rehydration affords a way of obtaining sections from blocks of tissue which have become hard in the process of embedding in paraffin, or which are inherently refractory to cutting. When hard tissues are encountered, the blocks can be trimmed so that one surface of the specimen is exposed, and soaked from a few hours to overnight in any one of the detergent solutions listed. Paraffin blocks will then yield excellent ribbons of sections, which are well preserved histologically, and which flatten smoothly when mounted.

Acknowledgment

This research was supported by a grant from the Biological Stain Commission.

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A DIFFERENTIAL STAINING METHOD FOR ELASTIC FIBERS, COLLAGENIC FIBERS, AND KERATIN

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Received for publication August 17, 1950

ABSTRACT.—**A method allowing for the differential presentation of *elastic* fibers, other connective tissue fibers, epithelial and other types of cytoplasm, and *keratin* is described. The procedure is based on the affinity of orcein for elastic fibers, of anilin blue for collagenic material, and of orange G for keratin. Bouin-fixed, tissue-mat embedded sections are stained in Pinkus' acid orcein for 1½ hours and rinsed in distilled water. The sections are differentiated in 50% alcohol containing 1% hydrochloric acid, washed in tap and then in distilled water. The sections are next transferred for 1 to 2 minutes to the anilin blue, orange G, phosphomolybdic acid combination known as solution No. 2 of Mallory's connective tissue stain, diluted 1:1 with distilled water. They are then rinsed in distilled water, quickly passed into 95% alcohol, and dehydrated in absolute alcohol containing some orange G, after which they are cleared and mounted. Within less than two hours sections may be stained and mounted with the following results: elastic fibers — red; collagenic fibers — blue; muscle fibers — yellow; keratin — orange.**

A project covering the prenatal and postnatal growth of wool and hair is now under way in the Bureau's Animal Fiber Laboratory at Beltsville, Maryland. A large number of animals are being used for this study and one of the first requirements was to find a specific stain that would enable us to differentiate elastic, collagenic and muscle fibers, to distinguish glandular and epithelial cytoplasm, and to recognize keratinized regions in the dermis.

Bouin's fixative was used throughout (picric acid, saturated aqueous solution 75 parts, formalin 25 parts, glacial acetic acid 5 parts). The skins studied were those of Karakul and hybrid sheep and common American goats, cut at or about 8 μ thick.

Various elastic fiber stains were considered, but none were promising inasmuch as we also insisted upon a characteristic color reaction for keratin. Mallory's connective tissue stain did not prove satisfactory for our purpose as the elastic fiber elements could not be clearly recognized.

Van Gieson's connective tissue stain was tried in combination with ferric chloride hematoxylin. It shows clear tinctorial differentiation between skin glands, connective tissue fibers, inner and outer

root sheaths, and the epidermis. The only objection to this otherwise excellent combination is that elastic fibers are not distinguishable from the collagenic fibers. However, it is recommended as an excellent nuclear and general connective tissue stain. The following procedure was found suitable:

1. Deparaffinize and hydrate the sections.
2. Stain 2 to 3 minutes in freshly made ferric chloride hematoxylin solution, and rinse in distilled water. The solution is made up as follows:

4.0% ferric chloride	2.0 ml.
2.0% hematoxylin	2.0 ml.
distilled water	46.0 ml.

3. Differentiate and counterstain quickly in a solution containing 40 parts of saturated aqueous picric acid to 1 part 1.0% aqueous acid fuchsin, and rinse in distilled water. It is well to control the differentiation under the microscope until only the nuclei show the grayish color of hematoxylin.
4. Dehydrate by dripping onto the slide 95% alcohol saturated with picric acid. The use of picric acid insures the bright yellow keratin stain. Clear and mount.

Successful preparations will show:

chromatin	black or gray
muscle	yellow
connective tissue	red
keratinized regions	bright yellow
cytoplasm	yellow

Differentiation of Connective Tissue Fibers and Keratin by Means of Orcein, Anilin Blue, Phosphomolybdic Acid, and Orange G

This new method is based on the principle of specific affinity of orcein for elastic fibers, aniline blue for collagen, and orange G for keratin. Staining is relatively simple and can be accomplished within two hours. The procedure is as follows:

1. Deparaffinize and hydrate the sections.
2. Stain for 1½ hours in the acid orcein of Pinkus,[1] and then rinse in distilled water. To make the orcein solution, 1.0 gram of the dye is dissolved in 100.0 ml. of 70% alcohol, to which 0.6 ml. of hydrochloric acid has been added.
3. Differentiate in 50% alcohol containing 1% hydrochloric acid. The exact time will vary, depending upon the material and the thickness of the sections. Control the differentiation under the

[1]Pinkus, Hermann. 1944. Acid orcein Giemsa stain (modification of Unna-Taenzer method). A useful routine stain for dermatologic sections. Arch. Dermat. and Syph., **49**, 355–6.

microscope until most of the pink is extracted from the sections. Wash thoroughly under tap water, and follow by a rinse in distilled water.

4. Transfer to Mallory's solution No. 2, diluted 1:1 with distilled water, for 1 to 2 minutes and then rinse in distilled water. Mallory's No. 2 solution is made up as follows:

Anilin blue	0.5 g.
Orange G	2.0 g.
1.0% solution of phosphomolybdic acid	100 ml.

5. Pass through 95% alcohol and finish dehydrating in absolute alcohol with orange G (1 part absolute to 1 part of a saturated solution of orange G in absolute). Clear in xylene and mount. If orange G accumulates excessively, pass through pure absolute alcohol prior to xylene.

Except for the orcein, the following stains were certified: hematoxylin, code No. CH–7; acid fuchsin, CR–7; anilin blue, CK–7; orange G, CO–7; orcein, an uncertified sample.[2]

Satisfactorily stained sections will show the following:

elastic fibers	red
collagen	blue
muscle fibers	yellow-orange to dirty yellow
cytoplasm	various shades of yellow
erythrocytes	golden yellow
keratinized material	bright orange

Although unsuited for critical chromatin studies, enough nuclear differentiation is obtained so that the nuclei of the sebaceous glands, ordinary metabolic nuclei, and the resolved-chromatic stages are clearly demonstrated.

[2]A personal communication from one of the authors names an American concern from which this orcein sample was obtained in 1944. At that time this company was actually importing its orcein from Europe; but at present no more orcein is available from this source. For the last few years no satisfactory orcein was on the market, so far as is known to the Stain Commission. Since this paper was received for publication, however, the situation has greatly improved. Certified orcein of American manufacture is now available, and is of good quality. *Ed.*

HISTOCHEMICAL COMPARISON OF THE CASELLA, BAUER, AND PERIODIC ACID OXIDATION-SCHIFF LEUCOFUCHSIN TECHNICS

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Received for publication October 21, 1950

ABSTRACT.—**Chromic acid, potassium permanganate and periodic acid apparently produce aldehyde from the same general group of substances. Chromic acid and potassium permanganate also destroy the aldehyde which they have produced, as well as that previously produced by periodic acid oxidation and by the Feulgen hydrochloric acid hydrolysis. Sulfite blockade, by occupying a considerable proportion of the aldehyde groups produced by periodic acid oxidation, creates a Schiff reaction similar to that produced by primary or secondary chromic acid or permanganate oxidation.**

It is suggested that the periodic acid Schiff positive substances which fail to give distinct Bauer and Casella reactions, are those with relatively few reactive 1,2 glycol, 1,2 OH,NH_2, or 1,2 OH,NHR groupings per molecule.

While the aldehyde reactions produced by oxidation with chromic acid, permanganates and periodic acids have been thought to be of essentially similar nature, there is an impressive list of differences of reaction to the three oxidant-Schiff technics of various tissue components. The list presented in table 1 is derived largely from our own experiences, principally because most other workers with the periodic acid and chromic acid Schiff reactions have not used the two tests in parallel on the same material.

A number of other substances recorded in the literature or in our own notes give positive reactions with the HIO_4 Schiff method, but are omitted from the table because no positive statements are available regarding their Bauer or Casella reactions. These include the E deficiency pigment of the rat uterus (Elftman, Kaunitz and Slanetz, 1949), Kurloff bodies (Pearse, 1949), intranuclear inclusions of the human vas deferens (McDonald, 1950), acrosomes and head caps of intratesticular spermatozoa (Elftman, 1950; Leblond, 1950; Wislocki, 1950; Clermont, 1950), egg albumen (Hotchkiss, 1948), a non-lipid component of the Golgi system (Gersh, 1949) and granules in rat lachrymal and mammary glands (Leblond, 1950). In addition the writer (1947a) has recorded the presence of Bauer positive polysaccharides which are digested slowly by diastase in *Klossiella muris* and *Toxoplasma sp.* and non-digestible Bauer positive material in the shells of the eggs of *Schistosoma mansoni,* in the opercular plugs of the eggs of *Capillaria (Hepaticola) hepatica,* as well as the presence of Bauer positive glycogen in various protozoa.

TABLE 1. SCHIFF REACTIONS OF TISSUE ELEMENTS AFTER HYDROLYSIS AND OXIDATION TECHNICS

Material	Source	Species	Casella 1% $KMnO_4$, 20′ Schiff	Bauer 5% CrO_3, 60′ Schiff	McManus HIO_4 10′ Schiff	Lillie HIO_4 10′ Schiff IH+Picric	Diast. HIO_4 Schiff
Desoxyribonucleic acid	Nuclei	General[1, 2]	–	–	–	–	–
Polysaccharides:							
Glycogen	Various	Various	RP	PR	RP	RP	–
Starch	Contents stomach and intestine; in vitro	Various	RP+	PR	RP++	RP++	RP to –
Cellulose		Various	PR	PR	PR	PR	PR
Agar	Used as imbedding matrix[1]		–	R	R	R	
Mucin: Epithelial	Saliv. Gld.	Various	–	PR	RP	RP	RP
	Conjunctiva	GP, Rab	PPk to –	PR	RP	RP	RP
	Bucco-phar. Glds.	Man, Rat, GP	Pk	PR	RP	RP	RP
	Bucco-phar. Glds.	Rabbit	–	– to Pk	– to Pk	– to Pk	– to Pk
	Gastric epith.	Man, Dog, Rodent	RP	PR	RP+	RP+	RP+
	Gastric Glds.	"	Pk to PR	PR±	PR	PR	PR
	Brunner Glds.	"	– to PPk	Pk to PR	PR	PR	PR
	Goblet Cells, Sm. Int.	"	– to Pk	PR	RP	RP	RP
	Colon Gld. Mouths	"	–	PR to –	RP to –	RP to –	RP to –
	Colon Gld. Bases	"	–	– to Pk	– to PPk	– to PPk	– to PPk
	Trachea, Bronchi	Man, Cat, Rodent	–	PR	RP	RP	RP
	Uterine Cerv. Gld.	Man	Pk to PR	PR	RP	RP	RP
	Vaginal Epith.	Rodent	– to PR	PR	RP	RP	RP
Mucin Connective Tissue	Vitreous	Dog, GP[1, 3] Rabbit	– to Pk	– to Pk	PR	PR	PR
	Umbil. Cord. Matrix	Man	–	–	–	–	–
	Umbil. Cord. Artery	Man	–	–	–	–	–
	Myxoma	Man	–	–		– to Pk.	

	Acute torulosis[1]	Rodent[1, 3]	–	–	PPk	PPk	PPk
Chordoma Mucin	Sacral	Man	–	– to PR	PR	PR	– to PR
Cyst and Follicle Fluid	Ovary	Man, Rodent	– to PR	PR	RP	RP	RP
Zona pellucida, Ova	"	Rodent	– to PR	PR	RP	RP	RP
Descemet's Membrane	Cornea	G.P., Dog, Rabbit[3]	PR to –	PR to –	RP	RP	RP
Lens Capsule	Eye	" [3]	PR	PR	RP+	RP+	RP+
Bone Matrix	Vertebra	Rodent	–	–		OR to O to Y	
Cartilage Matrix	Vert., Trachea	Man, Cat					
		Rodent	– to Pk	PR	PR	PR	PR
Nucleus pulposus	Intervert. disc	Rodent	–	–	– to Pk	– to Pk	– to Pk
Collagen	General, sclera	Various	– to Pk	–	PR	R-O-Y	+
Corneal Collagen	Eye	GP, Dog, Rabbit[1, 3]	– to Pk	– to Pk	PR	PR	PR
"Collacin" basophil Degen.	Skin	Man	–	–	Pk	Pk	Pk
Gelatin	Used as imbedding matrix					Obr	
Reticulum	Mucosae, Organs	Various[3]	–	–	PR	PR	PR
"	Smooth & Stri. Muscle	" [3]	–	–	PR	PR	PR
Basement Membranes	Capillary	" [1, 3]	– to P	–	PR	PR	PR
"Colloid"	Thyroid	Cat, Rodent.	Pk to PR	Pk to PR	RP	RP	RP
"Colloid"	Hypophysis	Man	PR to PR±	PR to Pk±	RP	RP	RP
Cyanophil Cells	"	"	GrPk	GrPk±	RP	RP	PR
Amyloid	Liver	" [1]	Pk	OY	Pk to PR	Pk to PR	Pk to PR
Chromaffin	Adrenal	Man, Rodent	– to Pk±	–	PR	PR	PR
Enterochromaffin	Intestine, Stomach	Man, Dog, Rodent[1, 3]	–	–	–	–	–
"Melanosis" pigment	Colon	Man	–	–	RP	RP	RP
"Enterosiderin"	Intestine	GP	–	–	br to RP	br to RP	br to RP
"Lipofuscin"	Adrenal	Man, GP	–	–	br to PR	br to PR	br to PR
Melanin	Skin, Chorioid, Retinal Epith.	Rodent, Dog[1, 3]	–	–	–	–	–
"Lipofuscin"	Heart Muscle	Man[1, 3]	br to Pk	br to –	br to Pk	br to Pk	br to Pk
Fibrin	Pneumonic exudate	Man	–	–	Pk to PR	OPk to OR	+
Plasma, Serum	Blood vessels	Various	–	–	Pk to PR	OPk to OR	+
Russell bodies	Inflammatory exudate	Man	– to Pk	– to Pk	RP	RP	RP
Hyaline casts	Renal tubules	Man, Rodents	– to RP	Pk to PR	RP	RP	RP

TABLE 1. *Concluded*

Material	Source	Species	Casella 1% $KMnO_4$, 20′ Schiff	Bauer 5% CrO_3, 60′ Schiff	McManus HIO_4 10′ Schiff	Lillie HIO_4 10′ Schiff IH+Picric	Diast. HIO_4 Schiff
"Brush border"	Renal tubules	Man, Rodents	–	Pk to PR	Pk to PR	Pk to PR	Pk to PR
Contents	Seminal Vesicle	Rat	Pk	– to PPk	RP	RP	
Contents	Prostatic glands	Man[1]	Pk	Pk to –	Pk to PR	Pk to PR	Pk to PR
Corpora amylacea	Prostate	Man[1]	Pk to PR	Pk	PR	PR	PR
Tissue mast cells		Rat	–	–	–	–	–
Tissue mast cells		Mouse	–	–	Few Pk to PR	Few Pk to PR	
Tissue mast cells		Man	–	–	Many PR	Many PR	
Zymogen granules	Pancreas	Rabbit, Man	–	–	RP	OR to RP	RP
" "	"	Dog, GP, Rat, Mouse	–	–	–	–	–
" "	Salivary Gld.	Man, Rodents	–	–	RP	RP	RP
" "	Paneth Cells	Man	–	–	–	–	–
" "		Rat, GP, Rabbit	–	–	PR	OR	+
Alpha granules	Hypophysis	Man	–	–	–	Y	Y
Eosinophil granules	Leucocytes	Monkey[4]		–	+		+
	Tissue leucocytes	Rat[1, 3]	–	–	–	–	–
Hemosiderin	Spleen	Man	–	–	–	–	–
Ceroid	Cirrhotic liver	Rat	– to RP	– to PR	RP	RP	RP
Kerasin	Gaucher's disease	Man	– to PK	– to PK	RP	RP	
Myelin	Brain, Cord	Various	–	–	–	–	–
Corpora Amylacea	Brain	Various	–	PR	PR	PR	PR
Acromeres of Rods	Retina	Dog, GP, Rabbit[3]	– to PR	– to PPk	Pk to RP	Pk to RP	Pk to RP
Bacterial chitin	Anthrax, Streptococ., Various intest. Bacilli[3]		–	–	RP	RP	RP
	Diphtheroids[3]		– to PPk	PPk	PR	PR	PR
	Mcb. tuberculosis[3]		–	–	–	–	–

Yeast chitins			GrP to −	PR	RP	RP	RP
Filaments	Granules of Actinomycosis	Man		R	RP	RP	RP
Filaments	Granules of Botryomycosis	Man	−	R	RP	RP	RP
Mold chitin	Aspergillosis	Rabbit	− to PR	R	R	R	R
Polysaccharide	Sarcocystis	Cat, Rodents	R	R	RP	RP	− to R
Megakaryocyte cyt.	Marrow	Rat	Pk	Pk	Pk	Pk	Pk
" "	"	GP	−	−	Pk	Pk	Pk
" "	"	Man[4]		+	+		−
" "	"	Monkey[4], Rabbit		−	+		+

In general the technics used for the study of materials in this table follow Lillie (1948), pp. 105, 143, 188. Symbols have the following significance:

P: purple	Y: yellow	No marks: moderate
R: red	br: brown	±: light
Pk: pink	−: negative	∓: faint
Gr: gray	++: very intense	
O: orange	+: intense	

[1]Direct Schiff reaction, without hydrolysis or oxidation, negative; in the case of "lipofuscin" the color is brown.
[2]Feulgen reaction positive.
[3]Feulgen reaction negative.
[4]From Rheingold and Wislocki (1948), Wislocki, Rheingold and Dempsey (1949).

While Bauer (1933) did not realize that he was dealing with a chromic acid oxidation, and referred to the process as hydrolysis, the Italian school of Vialli apparently recognized that an oxidation to aldehyde from hydroxyl was involved. Bignardi (1935) produced metachromasia by oxidation with sodium persulfate, but was not able to demonstrate aldehyde with Schiff reagent, and theorized that chromic acid produced first aldehyde, and then carboxyl groups, thus producing metachromasia as well as an aldehyde reaction, while the persulfate oxidized immediately to carboxyl. Casella (1942) in further exploration, found that permanganate would oxidize glycogen, mucin, and cartilage matrix to give an aldehyde reaction with Schiff reagent, but did not observe post oxidation metachromasia with this reagent. Hence he considered that permanganate carried the oxidation of the carbohydrate hydroxyls only to the aldehyde stage.

This work, published in German and Italian journals during the war period, did not come to our attention until after, at the suggestion of Claude Hudson, I had found both the permanganate and periodic acid oxidation reactions of glycogen, and recognized their parallelism with the Bauer chromic acid reaction (Lillie, 1947 a & b).

Both Hotchkiss, (1948), who discovered the periodic acid Schiff reaction in 1945, but did not report it until 1948, and Marchese (1947), commenting on the first report of McManus (1946) on the same method, recognized a probable parallelism between the new method and the Bauer chromic acid-Schiff sequence reaction. Both also based the histochemical use on the work of Jackson and Hudson (1937) who first applied the Malaprade reaction to hexose polysaccharides.

In our brief experience with the Arzac (1950) technic using a sequence fuchsin stain and sulfite treatment in place of the usual Schiff reagent treatment after periodic acid oxidation, it was observed (Lillie, 1950) that that method often failed to demonstrate collagenous and reticular connective tissue, and that similar results could be produced by interposing a sulfite reduction between the periodic acid oxidation and the Schiff aldehyde test. Atkinson (1950) remarked that a similar suppression of background staining could be achieved with Schiff reagent diluted 1:100 in bisulfite solution, and that glycogen, mucus and cartilage could be thereby demonstrated more conspicuously. The probable explanation of these findings was that with the interposed sulfite bath, and with the proportionately excessive amount of sulfite present in the sulfite step of the Arzac procedure and in the diluted Schiff reagent used by Atkinson, a considerable proportion of the free aldehyde groups present were converted into aldehyde sulfite complexes and thereby rendered unavailable to react with the Schiff sulfite leucofuchsin.

Experimental

In the following experiments a solution of potassium periodate (KIO_4) 0.8 g., 70% nitric acid 0.3 ml. and water 100 ml. was used as the periodic acid reagent; 5% aqueous chromic acid (CrO_3) and 0.5% aqueous potassium permanganate ($KMnO_4$) as the other two oxidant reagents. Schiff reagent for the aldehyde test was made as prescribed on p. 143 of Histopathologic Technic (Lillie, 1948). The aldehyde test was performed as directed on p. 144 (ibid.) for 15 minutes, except where otherwise stated. Three washes in M/20 $NaHSO_3$ totalling 4½ minutes were used directly after the Schiff reagent, and sections were then washed 10 minutes in running water, counterstained with iron hematoxylin and picric acid (ibid, p. 189), dehydrated, cleared and mounted through an alcohol, xylene, polystyrene sequence.

OXIDATION TESTS

The first question to be answered was this: Do collagen, reticulum, basement membranes, some mucins, intestinal "melanin", adrenal lipofuscin, chromaffin, etc., fail to yield positive Schiff aldehyde reactions after chromic acid or potassium permanganate oxidations because of a failure to convert the reactive 1,2 glycol, 1,2 OH,NH_2, and 1,2 OH,NHR groupings to aldehyde or because the aldehyde is formed in lesser quantity than from glycogen, cartilage, mucins, the lens capsule, etc., and then further oxidized to carboxyl or carbon dioxide, thus preventing a sufficient concentration of aldehyde to permit histochemical localization?

It was thought that if a chromic acid or a permanganate treatment were interposed between the standard periodic acid treatment and the application of the Schiff reagent, that any destruction of the aldehyde formed by the periodic acid would be manifested by weakening or abolition of the Schiff reaction of certain tissue elements as compared with the reaction of the same elements in the normal periodic acid Schiff method.

The material used was guinea pig liver, fixed in 5% acetic, 10% formalin alcohol for glycogen, guinea pig kidney fixed in Zenker's fluid, guinea pig adrenal fixed in Kose's bichromate formalin for chromaffin, guinea pig eye frozen in carbon dioxide and petroleum ether, opened while frozen and fixed at 0°C. in Spuler's formalin Zenker mixture, dog stomach fixed in acetic alcohol formalin and a human pseudoleukemic lymph node heavily infiltrated by eosinophil myelocytes and leukocytes.

Chromic acid treatments of 20 and 120 minutes and potassium permanganate treatments of 10 and 60 minutes were employed after 10 minutes in periodic acid and alone, and periodic acid oxidations of 10 minutes were practiced after 60 minute chromic acid and after 20 minute permanganate treatments.

Post oxidation of periodic acid preparations with chromic acid or with potassium permanganate weakens and then abolishes the

Schiff reaction of collagen and reticulum, and weakens the reaction of mucin, so that the final picture resembles that produced by chromic acid or permanganate alone. The chromic acid and permanganate pre-oxidation tried in this series did not destroy the ability of the tissues to yield normal periodic acid Schiff reactions. The permanganate oxidation appeared to greatly impair the stainability of normally oxyphil substances with picric acid, and to enhance somewhat their basophilia toward iron hematoxylin, while chromic acid impaired staining of nuclei and perhaps increased picrophilia.

In a second series of tests oxidation with periodic acid was prolonged to a 30 hour bath, without destroying the reaction. With chromic acid and with potassium permanganate treatment was terminated at 18 hours, because no further Schiff positive material could be found. The 6 hour permanganate oxidation likewise gave no Schiff positive material. Oxidation with periodic acid after 18 hours in chromic acid and after 6 hours in permanganate, yielded no Schiff positive material. Apparently the 6 hour $KMnO_4$ treatment is about marginal, as later tests have shown scattered denser connective tissue masses and hyaline casts which still reacted in a prevailingly negative field, using periodic acid after permanganate.

Illustrative data from the foregoing experiments are summarized in Table 2.

It is concluded from the foregoing experiments that periodic and chromic acids and potassium permanganate oxidize the same materials to the aldehyde state, and that the two latter destroy the aldehyde which they themselves form, and will destroy that formed by periodic acid as well.

When Feulgen reactions are performed after a 5 minute hydrolysis in normal hydrochloric acid preheated to and kept at 60°C., and a 2 hour oxidation by periodic acid, or 3% hydrogen peroxide is interposed before a 15 minute application of the Schiff reagent, the intensity of the nucleal reaction is unimpaired. Application of 5% chromic acid for the same period totally inhibited the nucleal reaction. Potassium permanganate weakened nuclear staining considerably, and sometimes destroyed it. A 1% ferric chloride solution had a similar effect.

SULFITE BLOCKADE TESTS

Further, the interposition of a 2 hour M/20 $NaHSO_3$ bath, further testing the aldehyde nature of the Feulgen reaction by sulfite blockade, inhibited the Schiff reaction.

When the Feulgen hydrochloric acid hydrolysis was prolonged to 10 minutes at 60°C., stronger Schiff reactions were obtained, even with only a 20 minute bath in Schiff reagent, and longer treatment with sulfite was required to suppress the Feulgen staining of less densely staining nuclei, while that of more strongly reacting nuclei was not suppressed.

TABLE II. EFFECT OF SEQUENCE DOUBLE AND PROLONGED SINGLE OXIDATIONS ON THE SUBSEQUENT SCHIFF REACTION OF VARIOUS TISSUE ELEMENTS

Sequence of reagents	A	A	A	A	A	A	B	B	B	B	C	C	C	C	C	AB	AB	AC	AC	BD	BD	CD	CD
A 0.8 HIO_4, 0.3 HNO_3	10′	30′	2h	6h	18h	30h										10′	10′	10′	10′				
B 5 CrO_3							20′	2h	6h	18h						20′	2h	—	—	1h	18h		
C 0.5 $KMnO_4$											10′	1h	2h	6h	18h	—	—	10′	1h	—	—	30′	6h
D 0.8 HIO_4, 0.3 HNO_3																				10′	10′	10′	10′
Glycogen, GP liver	5	6	5	5	4	5	3	4	2	0	3	4	3	0		5	3	5	4	5	0	5	0
Portal collagen	3	4	4	2x	2x	3x	0	0	0	0	2	1	0	0		3x	0	3	1	3x	0	3	0
Renal stroma, GP	4	4	4	4	2	1	0	0	0	0	0	2	0	0	0	2	0	4	3	4	0	4	0
brush borders	3	2	3	3	1	2	1	0	0	0	2	2	0	0	0	3	2	3	3	3	0	3	0
tube casts	4	4	3	3	3	2	3x	2x	0	0	3	4	1	0	0	3	3x	4	3	4	0	4	2
Adrenal chromaffin, GP	3	2	3		2	2	0	0	0	0	1	1	0	0	0	2	0	3	2	3	0	3	
reticulum	4	4	4		1	0	0	0	0	0	0	0	0	0	0	1	0	4	4	4	0	4	
lipofuscin	4	3	3		3	3	3	2	0	0	3	3	0	0	0	4	3	4	3	4	0	?4	
Eye, GP, Rod galactolipin	4	4	4	4	3	3	2	0	0	0	2	2	0	0	0	2	1	4	2	4	0	4	
lens caps. & Desc. memb.	5	5	5	4	4	5	3	3x	0	0	3	4	3	0	0	4	2	5	4	4	0	4	
scleral collag	3x	3x	3x	3x	2x	2	0	0	0	0	1	1	0	0	0	2x	2x	3	2	3x	0	4	
corneal collag	3–4	4	3	4	4	4	1	2	0	0	2	1	0	0	0	4	2	4	3	4	0	4	
Gastric surf. mucin, dog	6	5	5	5	5	6	3	3	0	0	3	1	0	0	0	5	3	6	4	6	0	6	
gland mucin	4	4	4	4	4	4	2	2	0	0	1	1	0	0	0	3	2	4	3	4	0	3	
reticulum, muscle	3	4	4	4	4	4	0	0	0	0	1	0	0	0	0	2	0	3	1	4	0	4	
Eosinophil myeloc., man	3	3x	3	3x	3	3	0	0	0	0	3	2	0	0	0	3x	0	3	2	3	0	3	
Appendix, collag., man	4	4	4	4	4	4	0	0	0	0	2	1	1	0	0	2	0	4	2	4	0	4	

0 yellow^x, unstained
1 pale pink or pale orange pink^x
2 purplish pink, orange pink^x
3 purplish red, orange red^x
4 red purple, red
5 deep red purple
6 very dark purple
x yellow tints due to acidophilia and the picric acid counterstain

Similarly a two hour M/20 bisulfite treatment of sections previously oxidized with periodic acid abolished the Schiff reaction, with a 20 minute Schiff reagent step, of collagen, bone matrix, megakaryocyte cytoplasmic granules, peripheral nerve stroma, salivary zymogen granules and duct contents, smooth and striated muscle reticulum (or sarcolemma ?) all renal stroma and the brush borders of the convoluted tubule epithelium, corneal and scleral connective tissue, thyroid colloid and Paneth granules. On the other hand glycogen in myelocytes, muscle and liver cells, mucin in the gastric epithelium and glands, in conjunctival and duodenal goblet cells and in Brunner glands, starch, cellulose, and yeast cell chitin, renal tube casts, the acromeres (ἄκρος = terminal, μέρος = portion) of retinal rods, the webwork in the vitreous, the lens capsule and Descemet's membrane and the cuticular border of the intestinal villi continued to react, although reactions were often distinctly less intense. In general parallel routine Bauer and Casella reactions on the same guinea pig, cat and rat tissues gave positive and negative results quite similar to those of the sulfite blocked periodic.

These findings seem to indicate again that the differences between the results of the routine periodic acid Schiff reaction and those of routine Bauer and Casella reactions are due to a lesser amount of remaining reactive aldehyde in those tissue elements which react negatively in the latter two reactions.

Since the foregoing series of tests was done with a 20 minute bath in Schiff reagent in place of the usual two hours prescribed for the Feulgen method (Lillie, 1948, p. 88–9) and the 15 minutes of the ordinary periodic acid reaction, it seemed necessary at this point to determine the effect of varying the Schiff bath time for the periodic acid reactions, and with and without sulfite blockade. Using cat trachea and thyroid, rabbit stomach, and guinea pig kidney, liver, duodenum and colon and a standard 10 minute oxidation in 0.8% KIO_4, 0.3% HNO_3 solution, it was determined that 5, 10, 15 and 20-minute baths in Schiff reagent gave essentially identical results, that after an interposed 30-minute blockade bath with M/20 $NaHSO_3$ a 2-hour bath in Schiff reagent gave as completely positive results as had the shorter Schiff treatment without intervening sulfite blockade, while shorter baths in Schiff reagent brought out fewer and fewer reacting elements so that the 10-minute Schiff treatment revealed red purple mucin, glycogen and cellulose, pale pink cartilage and renal casts and unstained renal brush borders, reticulum, basement membranes and collagen and almost unstained thyroid colloid. In a smaller parallel series of tests on Spuler-fixed human hypophyses it was found that sulfite treatment after periodic acid oxidation weakened greatly the positive reaction of the cyanophil cells and the colloid, and abolished that of the reticulum and capsular collagen.

ACETYLATION TESTS

It seemed that application of the acetylation and deacetylation technics of McManus and Cason (1950) before the Bauer and Casella reactions might afford further proof of their basic similarity to the periodic acid Schiff reaction. Preliminary trials indicated that 45 minutes at room temperature in a 40% acetic anhydride pyridine mixture was quite inadequate to block the periodic acid oxidation in our material. For the record the following failures are recorded: pure acetic anhydride 20 minutes at 25°C., 20% acetic anhydride in ethyl acetate 20 minutes 25°C., 10% acetic anhydride in pyridine 20 minutes at 25°C. and 20 and 60 minutes at 58°C. (this last destroyed the reactivity of renal stroma and brush borders, of reticular connective tissue generally, of appendix mucin and melanosis pigment but not that of gastric or duodenal mucin, starch, cellulose, glycogen or chromaffin), 20% acetic anhydride in pyridine 20 minutes at 25°C., 40% acetic anhydride in pyridine at 25°C. for 2 hours, 6 hours left gastric mucin, glycogen and chromaffin reactive. Finally 16 hours at room temperature in 40% acetic anhydride in pyridine was found sufficient to block the reaction of almost everything. Cartilage matrix alone gave a faint reaction, which was essentially lost at 18 hours acetylation. This discrepancy between McManus's results and ours may be assignable to higher room temperatures in Birmingham; our laboratory temperature was controlled fairly closely at 24–26°C. at the time. Even 18 hours acetylation left some starch granules and cellulose fragments HIO_4 Schiff positive.

Similarly deacetylation was found to be more difficult than McManus's results would indicate. The 45 minute saponification in N/10 KOH removed all the sections with and without collodionization. Similarly deacetylation in N/10 NaOH in 85% alcohol for 1, 2, 4, 8 and 24 hours resulted in loss of all sections. Finally use of 20 parts 28% ammonia and 80 parts 100% alcohol gave fairly successful deacetylation in 1 to 2 days at room temperature.

Accordingly sections of cat trachea, thyroid, parathyroid and esophagus fixed in Orth's fluid, of guinea pig adrenal fixed in Kose's fluid, and of dog eye fixed in 2% calcium acetate and 10% formalin after quick freezing in a solid carbon dioxide petroleum ether bath and blocking while frozen hard, were subjected to the following: 1. Control oxidation Schiff procedures. 2. The same preceded by 18 hours acetylation at 25°C. in 40 parts acetic anhydride to 60 parts pyridine by volume. 3. Acetylation as above followed by 24 hours at 25°C. in 20 parts 28% ammonia water to 80 parts absolute alcohol by volume, followed by the same oxidation Schiff procedures. The three oxidants compared were 0.8 g. KIO_4 : 0.3 ml. 70%HNO_3 : 100 ml. H_2O, for 10 minutes at 25°C.; 5 g. CrO_3 in 100 ml. H_2O for 30 minutes; and 0.5 g. $KMnO_4$ in 100 ml. H_2O for 20 minutes.

In the control series the usual positive reactions were obtained with all three oxidants with glycogen, mucin, cartilage matrix, thy-

roid colloid, the acromeres of the retinal rods, Descemet's membrane and the lens capsule, and positive reactions with periodic acid only on chromaffin, collagen, corneal fibrous tissue, muscle reticulum and vascular basement membranes. Positive reactions were generally weakest with chromic acid and strongest with periodic acid.

After acetylation, faint pink reactions with cartilage matrix were obtained with all three oxidants. Everything else failed to react.

After acetylation and deacetylation the previously recorded positive reactions of the control series were largely, but not completely, restored with all three oxidants.

SULFITE BLOCKADE TESTS OF CASELLA AND BAUER REACTIONS

Thus a further similarity of the three reactions is evident. That all three produce aldehyde from the acetylatable groupings is evident not only from the positive Schiff reaction engendered, but also from the fact that also the Casella and Bauer reactions may be blocked completely by interposition of a 4-hour treatment with M/10 $NaHSO_3$ after the oxidation step. The same sections of dog eye, cat trachea, thyroid and esophagus, and guinea pig adrenal were used for this test. This amount of sulfite treatment, followed by the same 10-minute bath in Schiff reagent, was sufficient to render negative the periodic acid Schiff reactions of corneal, scleral, tracheal, esophageal and adrenal capsule collagen, of adrenal and muscle reticulum and vascular basement membranes, of adrenal chromaffin and lipofuscin, and to weaken quite definitely the reactions of cartilage matrix, retinal rod acromeres, Descemet's membrane and the lens capsule, the parakeratin layer of the esophageal epithelium and thyroid colloid. Glycogen and tracheal gland mucin gave good positive reactions.

As with the periodic acid Schiff method, brief treatment of sulfite blocked sections with the same oxidant as had been used prior to the application of the 4-hour M/10 $NaHSO_3$ treatment, restored the positive Schiff reaction to those tissue elements which had given the reaction in the control series.

To restore the Bauer reaction, a 20-minute treatment with 5% CrO_3 was adequate, for the Casella a 15-minute immersion in 0.5% $KMnO_4$ and for the periodic acid Schiff 10 minutes in the usual KIO_4, HNO_3 mixture. The periodic acid Schiff reaction after partial blockade by a 4-hour treatment with M/10 $NaHSO_3$ was fully unblocked by 10-minute treatments with 3% H_2O_2, 1% $NaIO_3$, 1% $KClO_3$, the last two acidified with 0.3% HNO_3 1% $FeCl_3$ and by 2-minute treatments with 1% iodine, either as a diluted Lugol solution or as potassium hypoiodite. None of these solutions are capable of producing oxidation Schiff reactions of the substances reacting to periodic acid.

Discussion

From the foregoing it becomes evident that the use of chromic acid and potassium permanganate, either primarily as oxidants of

the reactive substances, or after the periodic acid oxidation, produces results quite similar to those produced by partial or complete sulfite blockade of the aldehyde groups formed by periodic acid. That the Bauer and Casella reactions depend on aldehyde formation is further confirmed by their complete blocking by sulfite after the oxidation step. That these oxidants destroy a considerable portion of the aldehyde which they form is shown by the greater ease of complete sulfite blocking as compared with the periodic acid method and the Feulgen HCl method, and by the fact that they greatly reduce the amount of demonstrable aldehyde preformed by the periodic acid and Feulgen technics when interposed between the phases of aldehyde formation and demonstration by color reaction.

Since the reactive groups involved in the three reactions appear to be the same, and the effect of chromic acid, permanganate, and sulfite treatment after periodic acid is to reduce the amount of aldehyde available to form the colored aldehyde sulfite leucofuchsin complex, it would appear that the then negatively reacting substances probably possessed fewer available 1,2 glycol, 1,2 hydroxy amine or 1,2 OH,NHR groupings per unit area than do such substances as glycogen, starch, cellulose, mucins and cartilage matrix. It is considered that the three polysaccharides possess one such grouping for each repeating unit. Chondroitin sulfuric acid, according to Meyer, Odier and Siegrist (1948) contains equal numbers of units of 1,5-glucuronic acid [D-glucuronic acid (1,5)] and 1,5-amino, 2-D-galactose [2-amino D-galactose (1,5)], totalling 60 pairs. They state that only the terminal units of the unbranched chains consume periodic acid. Hydrolysis also yields sulfuric and acetic acids. This much lower frequency of reactive groups accounts perhaps for the only bright purple red with Bauer and faint pink with Casella technics, and the lighter color with the periodic acid Schiff method than that yielded by glycogen.

Extending this suggestion to the epithelial mucins one might expect shorter chains for gastric mucin which is strongly positive and longer for colonic mucin which reacts relatively faintly. If metachromatic staining depends, as Lison (1936) has postulated, on sulfuric esters of high molecular weight, the greater and much more easily demonstrable metachromasia of colonic mucin, compared with the frequent unstainability of gastric surface mucin, would point in the same direction. And mast cell granules, which are the most intensely stained of metachromatically staining objects, are often difficult to demonstrate with the periodic acid Schiff method, especially in the rat, where the granules are perhaps the best stained with thionin.

Acknowledgments are due to Misses A. Laskey and J. Greco, to Mrs. H. J. Burtner and M. Duval and to Mr. Paul Jones for technical assistance.

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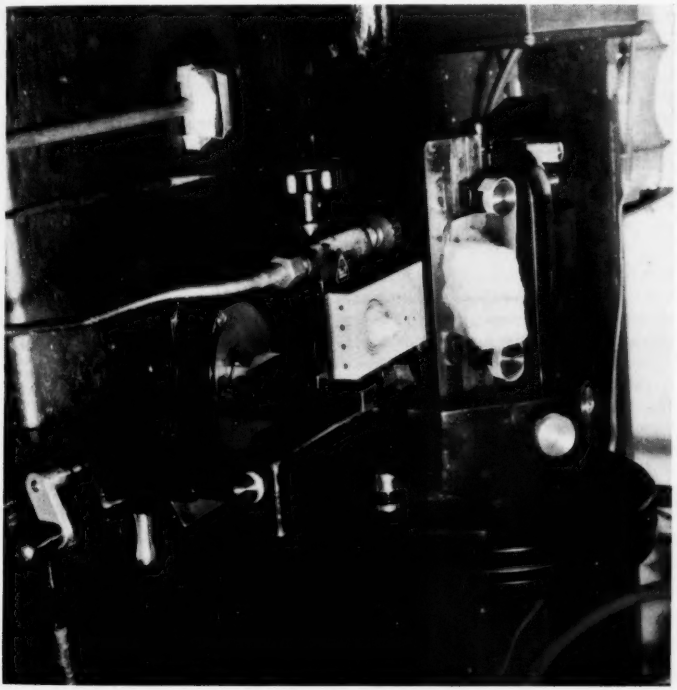
NOTES ON TECHNIC

An Improved Clinical Microtome for Sectioning Frozen Tissue

Adamstone and Taylor[1] have described a method for the rapid preparation of frozen tissue sections using a Leitz base sledge type microtome for the sectioning of frozen tissue. We have modified their technic of sectioning somewhat by using the modern Bausch and Lomb Improved Clinical Microtome (Catalog No. 31–81–76–03)



(Fig. 1). This microtome operates by having the knife move across the frozen tissue as opposed to the sledge type in which the mounted frozen tissue is moved while the knife is stationary. Difficulties in connecting the section mounting with a carbon dioxide tank are thus eliminated.

[1]Adamstone, F. B., and Taylor, A. B. 1948. The rapid preparation of frozen tissue sections. Stain Techn., **23**, 109–16.

. A fence (made from a piece of light copper or tin) is packed with dry ice and placed upon the knife in order to keep it cold. The other precautions and steps in technic are described in Adamstone and Taylor's paper.

We find that in using the sledge type microtome we are able to get satisfactory tissue sections only after numerous sectionings, and quite often the sections are of irregular thickness. By using the Bausch and Lomb Improved Microtome we are able to obtain almost an unbroken series of serial sections of 6–8μ thickness. The tissue sections are also of regular thickness throughout. – ROBERT T. WHITE and RUTH A. ALLEN, *Wilmer Ophthalmological Institute, Johns Hopkins University and Hospital, Baltimore, Maryland.*

A DEVICE FOR SIMULTANEOUS WASHING OF SMALL SPECIMENS

The handling of small specimens which have to be kept separately will always make the operations timewasting and difficult. Here is

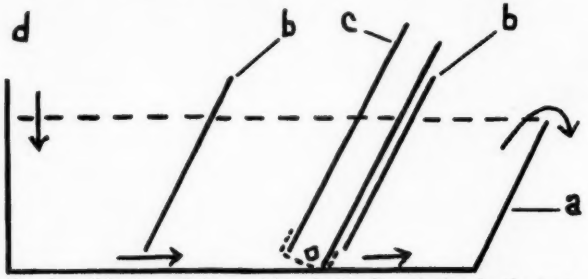


FIG. 1. – Side-view of the washing device. Description in the text.

described a simple device which during the last year has proved very satisfactory for the washing of small specimens in tap water. It consists of a rectangular box without lid. One of the shorter sides (a in the figure) is lower than the others and tilted a little. The box is divided into compartments by means of walls (b) parallel to the tilted one. These walls are as high as the three ordinary sides of the box, but they do not reach the bottom, leaving a little space free below. The specimens are placed in numbered tubes (c) the bottom of which is closed by a piece of coarse silk bolting or cheesecloth.

The tap water will enter the box at *d* in the figure and leave it over the lower side (a). The current will pass under the walls (c), and because of the tilted position of the walls there will be a sufficient exchange of the water in the tubes where the specimens are.

The box is easily cut out in celluloid in the size wanted and glued together with acetone. – A. S. JOHANSSON, *Zoological Laboratory, University, Oslo, Norway.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

BLANK, HARVEY, and McCARTHY, P. L. **A general method for preparing histologic sections with a water-soluble wax.** *J. Lab. & Clin. Med.*, **36,** *776–81.* 1950.

The recommended procedure is as follows: Wash and fix the tissues as usual. Impregnate with wax (Carbowax 4000, 9 parts and carbowax 1000, 1 part) for 3 hr. at 55°C. Transfer to a mold, fill with wax and cool in the refrigerator. Attach to a block and cut sections (serial if desired). Place on "floating solution" ($K_2Cr_2O_7$ 0.2 g., gelatin 0.2 g., distilled water 1000 ml.). Stain, dehydrate and mount. The method is simple, rapid and preserves excellent cytologic detail.—*John T. Myers.*

LATTA, HARRISON, and HARTMAN, J. F. **Use of a glass knife in thin sectioning for electron microscopy.** *Proc. Soc. Biol. and Med.*, **74,** *436–9.* 1950.

Glass microtome knives were made from a piece of plate glass about ⅜-inch thick in the following manner. A strip of glass 1½ inches wide and 12 inches long was scored for breaking with parallel lines running at an angle of 45° with the strip. Breaking the strip thus yielded a series of diamond-shaped pieces whose edges at the acute angles met to form the cutting edge. A special knife holder to fit a Spencer rotary microtome was constructed to hold the glass knife, and the angle of the cutting edge was adjusted to give a clearance angle of 10° between the vertical surface nearest the tissue block and the plane of travel of the block. This adjustment was made by rotating the knife holder in the usual manner for adjusting the tilt of a regular knife. For cutting very thin sections, the thermal expansion method of feeding the block into the knife was used. The block was cooled with a jet of CO_2 from a tank and the knife adjusted for contact with the block. The regular feed mechanism of the microtome was disengaged and several sections cut during the expansion of the block. The durability of the glass edge compared favorably with one of steel. A figure showing the apparatus and two illustrations of photographs taken at ×7000 and ×14,000 are included.—*H. A. Davenport.*

DYES AND THEIR BIOLOGICAL USES

GITLIN, DAVID. **Distribution of azo-proteins in the tissues of the normal mouse.** *Proc. Soc. Biol. and Med.*, **74,** *138–42.* 1950.

Colored compounds of the type, R-salt-azo-benzidine-azo-protein were prepared and purified and injected intravenously into mice (0.5 ml. of solution, containing 0.75 to 2.00 mg. azo-protein). Some animals were sacrificed at the end of 1 hr. and another group at the end of 28 hr. The distribution of the colored protein in organs, lymph nodes and macrophages was studied after embedding in paraffin, sectioning and mounting. Ovalbumin, gelatin and casein were used as the antigenic component, and aniline blue, safranin, alizarin red, acriflavine and neutral red in addition to R-salt for the colored component. The conclusions were that the distribution in the tissues was influenced both by the kind of protein and by the dye. The antigens were found in liver, kidney, intestine and lymph nodes, but not heart, lungs and brain. Traces were found in muscle.—*H. A. Davenport.*

McGREW, F. G., and SCHNEIDER, A. K. **The sorption of acid dyes on nylon.** *J. Amer. Chem. Soc.*, **72,** *2547–52.* 1950.

At moderate pH levels, there was evidence that a salt-type linkage occurred between the basic amine ends of the nylon polymer chain and the sulfonic acid groups of the dye. The affinity of dye for the fiber was measured quantitatively. In aqueous solution, there was no simple correlation between affinity and dye structure in regard to molecular weight, number of sulfonic acid groups, or molecular structure (azo and anthraquinone types). There was also no correlation between affinity (free-energy of sorption) and rate of sorption or penetration of dyes. Affinity was but one factor in sorption rate together with density or porosity of the polymer and the molecular size of the dye molecules.

Certain dyes inhibited absorption of others, and even replaced them on predyed fiber. For example, fiber dyed with anthraquinone blue B could be made yellow by digestion in a solution of Milling yellow G, but the reversal could not be carried out. A sequence was established in which each dye would replace any dye of lower order, but could not readily be replaced by any of the lower order dyes. The sequence coincided exactly with the arrangement of dyes in diminishing magnitude of affinity. The results indicate why the absorption of dyes on nylon from mixed solutions is accompanied by more irregularities than is the case with a polymer like wool. The smaller number of available basic sites in nylon leads to competition between dyes.—*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

MARSHALL, HOMER C., STOUGHTON, R. B., and KIRSNER, J. B. **Polysaccharides in ulcerative colitis and normal human rectum compared by stains of biopsy specimens.** *Proc. Soc. Exp. Biol. and Med.*, **74,** *498–500.* 1950.

The validity of the hypothesis that lysozyme plays a role in the pathogenesis of ulcerative colitis depends on the demonstration of a lysozyme substrate in the colon. Surface growth of *Micrococcus lysodeikticus* gave a strongly positive reaction for carbohydrate when tested by the McManus or the Hotchkiss method for polysaccharides. The positive reactions were obtained after embedding in paraffin and fixation by either formalin, alcohol or acetone. However, when the deparaffinized sections containing the organisms were subjected to the action of lysozyme, subsequent stains were negative. Lysozyme had no effect on the polysaccharide substances in the sections of either normal or pathological human colons. Even though the amount of lysozyme in the feces of patients suffering from ulcerative colitis is greater than that in normals, a direct relationship between the enzyme and the cause of the disease is not established.—*H. A. Davenport.*

STEINBERG, DANIEL, and SILVERSTONE, BERTRAM. **Radioautography of cerebral tumors employing P–32.** *Proc. Soc. Exp. Biol. and Med.*, **74,** *304–8.* 1950.

Previous investigation by Silverstone and Solomon (Trans. Amer. Neurol. Assoc., pp. 115–9, 1948) has indicated that radioactive phosphorus given intravenously to patients with brain tumor became concentrated in the tumor to values of 5 to 100 times its concentration in normal brain. Radioautography of sections taken from tumors removed at operation was employed to study this phenomenon. Frozen sections 25μ thick were allowed to dry on gelatin coated slides and then covered with a strip of Eastman No-screen X-ray film. The film was pressed against the section with another slide and bound with cellulose tape. The completed preparation was wrapped in black paper and stored in the cold room for an exposure period of 7–20 days. Each film was processed in 50 ml. of fresh Kodak D–19 developer for 5 min. at 20°C. Rigorous standardization of the development was necessary to insure valid comparison of autographs from different cases. Sections of 8μ immediately preceding and following the one used for autography were stained with hematoxylin and eosin for histologic orientation. Densiometric

measurements of the autographs showed the content of the isotope in tumor tissue to be much higher than in normal brain. This observation coupled with the information gained from chemical analyses of normal brain and of tumor tissue indicate that increased metabolic activity in the uptake of phosphorus is present in the tumors.—*H. A. Davenport.*

RICHARDS, OSCAR W., and GRAEF, IRVING. **Acid-fast membranes of lipid pneumonia by fluorescence microscopy.** *Proc. Soc. Exp. Biol. and Med.,* **74,** *121–3.* 1950.

Lung tissue from cases of pneumonia following the aspiration of unsaturated oil were embedded in paraffin, sectioned, mounted and stained in a 0.1% solution of auramin O (C. I. No. 655) dissolved in 3% phenol (aq.) for 2–5 min. The sections were then destained in a mixture of 0.5 g. NaCl and 1 ml. of HCl (conc.) in 100 ml. of 70% alcohol for 5–10 min. with at least two changes of fluid to remove the dye from all structures except the acid-fast ones. Thioflavine TG (C. I. No. 815) may be used instead of auramin. Applying a cover glass with balsam is undesirable because balsam is fluorescent and also causes fading. Polyvinyl alcohol without a cover glass is recommended. The brightest fluorescence is obtained from an arc or high amperage tungsten filament filtered through a blue-ultraviolet transmitting filter with a complementary yellow filter in or on the eyepiece. The acid-fast material is bright yellow on a yellowish background and is seen best in a room having subdued illumination. A Corning No. 5860 filter may be used with a mercury arc. Fluorescence microscopy is recommended as being more sensitive for revealing lipo-protein combinations of the acid-fast type than bright field microscopy with Ziehl-Neelsen staining.—*H. A. Davenport.*

WACHSTEIN, M. **Reduction of potassium tellurite by living tissues.** *Proc. Soc. Exp. Biol. and Med.,* **72,** 175–8. 1949.

The reactions of potassium tellurite and neotetrazolium chloride (Pannone Chemical Co., Farmington, Conn.) in fresh tissues and exudates of animal origin were compared. Sites of reducing activity were well shown with both reagents in rabbit kidney and in organs from rat and operatively removed tissue from man. Yeast and anaerobic bacteria also gave reducing reactions. Staining in the block was secured by immersing fresh tissue in 0.1% potassium tellurite in M/10 phosphate buffer at pH 7.4 for 8–12 hours previous to fixation. Results after paraffin embedding were inferior to frozen sections. Intravital reduction was demonstrated by injecting rats intraperitoneally, daily for 16 days with 0.8 ml. of either the tellurite solution or a saturated solution of neotetrazolium in buffer, and subsequent examination of the kidneys. Reduction occurred in the proximal convoluted tubules, but the vital staining was less intense than that seen after supravital staining. Active SH-groups appeared to be essential for the reduction of tellurite. Immersion of fresh kidney in a 0.1% solution of iodoacetamide for 1 hour blocked the reaction. (See Pratt and Dufrenoy, Stain Techn., **23,** 137–41, 1948, for a report of similar reactions in plant tissues.)—*H. A. Davenport.*

WACHSTEIN, M. **Histochemical demonstration of reducing activity in normal and leukemic blood and bone marrow cells.** *Proc. Soc. Exp. Biol. and Med.,* **73,** 306–8. 1950.

Previous observations (Wachstein, M., Proc. Soc. Exp. Biol. and Med., **72,** 175, 1949), that leucocytes from various exudates reduced both potassium tellurite and *pp* diphenylene bis 2-(3,5 diphenyl tetrazolium chloride), were extended to include normal and abnormal blood and bone marrow cells. The reaction given by the "neotetrazolium chloride" was more readily visualized than that given by tellurite. The sites of reduction were indicated by small pulpish granules of insoluble formazan. The blood or bone marrow suspension was added to a freshly prepared saturated solution of the neotetrazolium in M/10 aqueous phosphate

buffer and incubated at 37° C. Staining appeared to reach a maximum in about 1 hr. All white blood cells showed a positive reaction but myeloblasts were negative. Abnormal cells of chronic lymphatic leukemia varied from all negative to 98% positive. In surgically removed lymph nodes, 46–54% of the lymphocytes were positive. In bone marrow, the majority of the positively reacting cells were found in the myeloid series. Polymorphonuclear cells of the bloods of rats, guinea pigs, rabbits and mice reacted positively. Previously observed positive sites of reaction in tissue cells seemed to be closely associated with the mitochondria, but these granules did not seem to be responsible for the sites of reduction in blood cells.—*H. A. Davenport.*

WOLMAN, M. **Application of the Feulgen reaction to the laboratory diagnosis of smallpox.** *Proc. Soc. Exp. Biol. and Med.*, **74,** *85–7.* 1950.

Smears obtained from scrapings of the bases of lesions of variola and of varicella were fixed in a 1:1 alcohol-ether mixture, allowed to dry, and stained by the Feulgen technic. In the smears of variola, positively staining granules averaging about 0.3μ in diameter were seen both intra- and extracellularly. In the smears of varicella, however, no comparable granules were demonstrable. The use of the Feulgen staining technic is recommended as a laboratory procedure for the differential diagnosis between smallpox and chickenpox. The method was applied successfully also to the staining of smears and sections of chorioallantois cultures of smallpox virus and rickettsiae.—*H. A. Davenport.*

ZWEIFACH, B. W., BLACK, M. M., and SHORR, E. **Histochemical alterations revealed by tetrazolium chloride in hypertensive kidneys in relation to renal VEM mechanisms.** *Proc. Soc. Exp. Biol. and Med.*, **74,** *848–54.* 1950.

In the normal kidney the renal vasoexcitatory material (VEM) is produced by the cortex only under conditions of anaerobiosis, whereas the hypertensive kidney forms VEM under both aerobic and anaerobic conditions. A comparison of the nature of the water-insoluble formazan precipitated in slices of kidney treated by immersing them in a 0.5% solution of 2,3,5–triphenyl tetrazolium chloride in physiological saline or Ringer phosphate at pH 7.4, and incubating (37.5°C.) for 0.5 hr. was made. Kidneys from normotensive dogs showed the formazan deposit as fine dust-like granules throughout the cytoplasm of cells in the proximal convoluted tubules. Kidneys from hypertensive animals gave a much coarser deposit in which the formazan took the form of angular crystals, coarse needles and platelike granules. Similar results were obtained from kidneys of normotensive and hypertensive humans. Normotensive kidney subjected to 90 min. of anaerobiosis and then treated with triphenyl tetrazolium chloride under aerobic conditions gave a staining picture similar to that obtained with hypertensive kidney. Concurrent determinations of the oxygen consumption and of the total amount of formazan per unit weight showed no significant differences between normal and hypertensive kidney tissue.—*H. A. Davenport.*

MICROÖRGANISMS

LEONARDI, G. **La variazioni del contenuto citoplasmatico in acido ribonucleico durante la fasi evolutive della cellula batterica.** *Bol. Soc. Ital. Biol. Sper.*, **26,** *293–5.* 1950.

Histochemical technics, such as staining with Giemsa, agree with quantitative analysis in showing that in colonies of *Vibrio cholerae,* ribonucleic acid decreases with aging, while desoxyribonucleic acid remains nearly constant, representing 2 to 4% of the dry weight.—*J. Dufrenoy.*

LEONARDI, G. **Il significato biochimico del metodo di Robinow per la localizzazione degli acidi ribonucleico e dessoci-ribonucleico nella cellula batterica.** *Bol. Soc. Ital. Biol. Sper.*, **26,** *291–3.* 1950.

Treatment with 1*N* HCl for 10 min. at 60°C. extracts ribonucleic acid from *Vibrio cholerae,* without affecting desoxyribonucleic acid.—*J. Dufrenoy.*

MITCHISON, J. M. **Birefringence of Amoeba.** *Nature,* **166,** *313–14.* 1950.

Birefringence of *Amoeba proteus* membrane is decreased by fixing in 4% formalin for 10 min. and then immersing in a solution of potassium mercuric iodide of refractive index 1.56. This implies that the membrane has positive form birefringence and negative intrinsic birefringence. There are no striking differences in the birefringence of different regions of the membrane of a moving amoeba. Birefringence is found to be increased by staining with certain dyes but since the birefringence of dyes has not been fully investigated the results cannot be interpreted.—*G. Crossmon.*

PATEL, M. K., KULKARNI, N. B., GAEKWAD, S. R. **Staining bacterial flagella.** *Current Sci. (India),* **19,** *322–3.* 1950.

A loopful of 12-18-hr.-old culture grown on potato-dextrose agar is placed in a drop of water on a slide and spread on an area of 2 sq. cm. After air-drying, it is covered with 4 drops of solution A (2% tannic acid in distilled water, filtered; may be stored) and immediately followed by 4 drops of solution B (2 g. iodine dissolved in 10 ml. *N* NaOH and made up to 100 ml. with water). It is heated gently to steaming, without boiling, for about 2 minutes. Two drops of solution C (10 ml. 3% basic fuchsin in 95% alcohol added to 100 ml. 5% phenol) are added and the slide heated for 3 min. until steaming occurs. The slide is washed in a gentle stream of water and flooded with solution C again, heating for 1½ to 2 min., or until steaming. It is washed with water, dried and examined. Iodine and NaOH stand out, probably because of alkaline nature. Phenol, in solution C, intensifies basic fuchsin, possibly due to withdrawal of water from the cells and forming a shining metallic precipitate. Because of the fine precipitate a cleaner background is obtained than with other methods, and there is less interference with the staining of bacterial cells.—*A. E. Wasserman. (Courtesy of Biological Abstracts.)*